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(51) International Patent Classification 5 :		(11) International Publication Number:	**WO 92/03918**
A01H 1/00, C07H 21/00	**A1**	(43) International Publication Date:	19 March 1992 (19.03.92)

(54) Title: TRANSGENIC NON-HUMAN ANIMALS CAPABLE OF PRODUCING HETEROLOGOUS ANTIBODIES

(57) Abstract

The invention relates to transgenic non-human animals capable of producing heterologous antibodies, i.e., antibodies en-
coded by immunoglobulin heavy and light chain genes not normally found in the genome of that species of non-human animal.
In one aspect of the invention, transgenes encoding unrearranged heterologous human immunoglobulin heavy and light chains
are introduced into a non-human animal thereby forming a transgenic animal capable of producing antibodies encoded by hu-
man immunoglobulin genes. Such heterologous human antibodies are produced in B-cells which are thereafter immortalized, e.g.,
by fusing with an immortalizing cell line such as a myeloma or by manipulating such B-cells by other techniques to perpetuate a
cell line capable of producing a monoclonal heterologous antibody. The invention also relates to heavy and light chain immunog-
lobulin transgenes for making such transgenic non-human animals as well as methods and vectors for disrupting endogenous im-
munoglobulin loci in the transgenic animal. The invention also includes methods to generate a synthetic immunoglobulin variable
region gene segment repertoire used in transgene construction and methods to induce heterologous antibody production using an-
imals containing heterologous rearranged or unrearranged heavy and light chain immunoglobulin transgenes.

TRANSGENIC NON-HUMAN ANIMALS CAPABLE OF PRODUCING HETEROLOGOUS ANTIBODIES

TECHNICAL FIELD

The invention relates to transgenic non-human animals capable of producing heterologous antibodies, transgenes used to produce such transgenic animals, immortalized B-cells capable of producing heterologous antibodies, methods and vectors for disrupting endogenous immunoglobin loci, methods to generate a synthetic immunoglobulin variable region gene segment repertoire, and methods to induce heterologous antibody production.

BACKGROUND OF THE INVENTION

One of the major impediments facing the development of in vivo applications for monoclonal antibodies in humans is the intrinsic immunogenicity of non-human immunoglobulins. Patients respond to therapeutic doses of rodent monoclonal antibodies by making antibodies against the rodent immunoglobulin sequences. These human anti-mouse antibodies (HAMA) neutralize the therapeutic antibodies and can cause acute toxicity. The HAMA response is less dramatic in immunodeficient patients. Therefore, intrinsic immunogenicity has not prevented the use of rodent monoclonal antibodies for the treatment of graft rejection, which involves the temporary attenuation of the patient's immune response. Rodent antibodies may also be useful for treating certain lymphomas that involve immunodeficiencies. However, even immunodeficient patients can mount a HAMA response which leads to a reduction in safety and efficacy.

The present technology for generating monoclonal antibodies involves pre-exposing, or priming, an animal (usually a rat or mouse) with antigen. This pre-exposure leads to the formation of splenic B-cells that secrete immunoglobulin molecules with high affinity for the antigen. Spleen cells from a primed animal are then fused with myeloma cells to form

immortal, antibody secreting, hybridoma cells. Individual hybridoma clones are screened to identify those cells producing immunoglobulins directed against a particular antigen.

The genetic engineering of individual antibody genes

5   has been proposed. Two genetic engineering approaches have been reported: chimeric antibodies and complementarity-determining-region (CDR) grafting. The simplest approach, chimeric antibodies, takes advantage of the fact that the variable and constant portions of an antibody molecule are

10   encoded on separate exons. By simply fusing the variable region exons of a rearranged mouse antibody gene with a human constant region exons, a hybrid antibody gene can be obtained (Morrison, S.L., et al. (1984), Proc. Natl. Acad. Sci. USA, 81, 6851-6855). The major problem with this approach is that while

15   the highly immunogenic mouse Fc region is eliminated, the remaining mouse Fab sequences are still immunogenic (Bruggemann, et al. (1989), J. Exp. Med., 170, 2153-2157). The CDR grafting approach uses computer modeling to generate a completely artificial antibody in which the only mouse

20   sequences are those involved in antigen binding (Riechmann, L., et al. (1988) , Nature, 332, 323-327). Each of these approaches requires the prior characterization of a rodent monoclonal antibody directed against the antigen of interest, and both require the generation of a stable transfected cell

25   line that produces high levels of the engineered antibody.

Another approach to the production of human antibodies is a proposal involving the construction of bacterial expression libraries containing immunoglobulin cDNA sequences (Orlandi, et al. (1989), Proc. Natl. Acad. Sci. USA, 86,

30   3833-3837, and Huse, et al. (1989), Science, 246, 1275-1281). This technique reportedly has only been used to generate antibody fragments derived from mouse cDNA sequences.

A number of experiments have reported the use of transfected cell lines to determine the specific DNA sequences

35   required for Ig gene rearrangement (reviewed by Lewis and Gellert (1989), Cell, 59, 585-588). Such reports have identified putative sequences and concluded that the accessibility of these sequences to the recombinase enzymes

used for rearrangement is modulated by transcription (Yancopoulos and Alt (1985), Cell, 40, 271-281). The sequences for V(D)J joining are reportedly a highly conserved, near-palindromic heptamer and a less well conserved AT-rich

5   nanomer separated by a spacer of either 12 or 23 bp (Tonegawa (1983), Nature, 302, 575-581; Hesse, et al. (1989), Genes in Dev., 3, 1053-1061). Efficient recombination reportedly occurs only between sites containing recombination signal sequences with different length spacer regions.

10   The production of transgenic mice containing various forms of immunoglobulin genes has also been reported. Rearranged mouse immunoglobulin heavy or light chain genes have been used to produce transgenic mice. Such transgenes reportedly are capable of excluding the rearrangement of

15   endogenous Ig genes. See e.g. Weaver et al. (1985), Cell, 42, 117-127; Iglesias, et al. (1987), Nature, 330, 482-484; Storb et al. (1985), Banbury Reports, 20, 197-207; Neuberger et al. (1989), Nature, 338, 350-352; Hagman et al. (1989), J. Exp. Med., 169, 1911-1929; and Storb (1989) in Immunoglobulin Genes,

20   Academic Press, T. Honjo, F.W. Alt and T.H. Rabbitts eds. pp. 303-326. In addition, functionally rearranged human Ig genes including the μ or $\gamma 1$ constant region have been expressed in transgenic mice. Yamamura, et al. (1986), Proc. Natl. Acad. Sci. USA, 83, 2152-2156; Nussenzweig, et al. (1987), Science,

25   236, 816-819. In the case of the μ rearranged heavy chain gene, allelic exclusion of endogenous immunoglobulin gene loci was reported.

            Allelic exclusion, however, does not always occur in all transgenic B-cells. See e.g. Rath, et al. (1989), J.

30   Immunol., 143, 2074-2080 (rearranged μ gene construct); Manz, et al. (1988), J. Exp. Med., 168, 1363-1381 (μ transgenes lacking transmembrane exons did not prevent rearrangement of the endogenous genes); Ritchie, et al. (1984), Nature, 312, 517-520 and Storb, et al. (1986), Immunol. Rev., 89, 85-102

35   (transgenic mice expressing rearranged κ transgene capable of forming stable heavy/light chain complex only rearrange endogenous κ genes in B-cells that fail to correctly rearrange endogenous heavy chain gene); and Manz, et al. (1988), J. Exp.

4

Med., 168, 1363-1381 (transgenic mice containing κ gene
encoding light chain incapable of combining with heavy chains,
show only a low level of allelic exclusion). See also
Nussenzweig, et al. (1988), Nature, 336, 446-450); Durdik, et
5   al. (1989), Proc. Natl. Acad. Sci. USA, 86, 2346-2350; and
Shimizu, et al. (1989), Proc. Natl. Acad. Sci. USA, 86,
8020-8023.

        Somatic mutation has also been reported in a 15 kb
mouse κ gene construct in hyperimmunized transgenic mice
10   (O'Brien, et al. (1987), Nature, 326, 405-409; Storb (1989) in
Immunoglobulin Genes, Academic Press, T. Honjo, F.W. Alt, and
T.H. Rabbitts, eds. pp. 303-326) and in the variable portion of
a μ heavy chain transgene (Durdik, et al. (1989), Proc. Natl.
Acad. Sci. USA, 86, 2346-2350).

15   Ig gene rearrangement, though studied in tissue
culture cells, has not been extensively examined in transgenic
mice. Only a handful of reports have been published describing
rearrangement test constructs introduced into mice [Buchini, et
al. (1987), Nature, 326, 409-411 (unrearranged chicken λ
20   transgene); Goodhart, et al. (1987) , Proc. Natl. Acad. Sci.
USA, 84, 4229-4233) (unrearranged rabbit κ gene); and
Bruggemann, et al. (1989), Proc. Natl. Acad. Sci. USA, 86,
6709-6713 (hybrid mouse-human heavy chain)]. The results of
such experiments, however, have been variable, in some cases,
25   producing incomplete or minimal rearrangement of the transgene.

        Based on the foregoing, it is clear that a need exists
for heterologous monoclonal antibodies, e.g. antibodies of
human origin, derived from a species other than human. Thus,
it is an object of the invention herein to provide a source of
30   monoclonal antibodies that may be used therapeutically in the
particular species for which they are designed.

        In accordance with the foregoing object transgenic
nonhuman animals are provided which are capable of producing a
heterologous antibody, such as a human antibody.

35   Further, it is an object to provide B-cells from such
transgenic animals which are capable of expressing heterologous
antibodies wherein such B-cells are immortalized to provide a

source of a monoclonal antibody specific for a particular
antigen.

In accordance with this foregoing object, it is a
further object of the invention to provide hybridoma cells that
are capable of producing such heterologous monoclonal
antibodies.

Still further, it is an object herein to provide
heterologous unrearranged and rearranged immunoglobulin heavy
and light chain transgenes useful for producing the
aforementioned non-human transgenic animals.

Still further, it is an object herein to provide
methods to disrupt endogenous immunoglobulin loci in the
transgenic animals.

Still further, it is an object herein to provide
methods to induce heterologous antibody production in the
aforementioned transgenic non-human animal.

A further object of the invention is to provide
methods to generate an immunoglobulin variable region gene
segment repertoire that is used to construct one or more
transgenes of the invention.

The references discussed herein are provided solely
for their disclosure prior to the filing date of the present
application. Nothing herein is to be construed as an admission
that the inventors are not entitled to antedate such disclosure
by virtue of prior invention.

SUMMARY OF THE INVENTION

In accordance with the foregoing objects, in one
aspect of the invention, transgenic non-human animals are
provided that contain rearranged, unrearranged or a combination
of rearranged and unrearranged heterologous immunoglobulin
heavy and light chain transgenes in the germline of the
transgenic animal. For each of the foregoing animals,
functionally rearranged heterologous heavy and light chain
immunoglobulin transgenes are found in the B-cells of the
transgenic animal.

Heterologous heavy and/or light unrearranged
immunoglobulin transgenes are introduced into a host non-human

animal to produce a transgenic non-human animal containing a
heavy and a light heterologous immunoglobulin gene or an
intermediate animal containing one or the other transgene.
When incorporated into the germline of such intermediate

5   animals, crosses between one containing a heavy chain transgene
and one containing a light chain transgene produces a
transgenic non-human animal containing both heavy and light
heterologous immunoglobulin transgenes.

        The transgenes of the invention include a heavy chain

10 transgene comprising DNA encoding at least one variable gene
segment, one diversity gene segment, one joining gene segment
and one constant region gene segment. The immunoglobulin light
chain transgene comprises DNA encoding at least one variable
gene segment, one joining gene segment and one constant region

15 gene segment. The gene segments encoding the light and heavy
chain gene segments are heterologous to the transgenic
non-human animal in that they are derived from, or correspond
to, DNA encoding immunoglobulin heavy and light chain gene
segments from a species not consisting of the transgenic

20 non-human animal. In one aspect of the invention, the
transgene is constructed such that the individual gene segments
are unrearranged, i.e., not rearranged so as to encode a
functional immunoglobulin light or heavy chain. Such
unrearranged transgenes permit recombination of the gene

25 segments (functional rearrangement) and somatic mutation of the
resultant rearranged immunoglobulin heavy and/or light chains
within the transgenic non-human animal when exposed to antigen.

        In one aspect of the invention, heterologous heavy and
light immunoglobulin transgenes comprise relatively large

30 fragments of unrearranged heterologous DNA. Such fragments
typically comprise a substantial portion of the C, J (and in
the case of heavy chain, D) segments from a heterologous
immunoglobulin locus. In addition, such fragments also
comprise a substantial portion of the variable gene segments.

35   In an alternate embodiment, HP LaserJet Series
IIHPLASEII.PRSegments. In such transgene constructs, the
various regulatory sequences, e.g. promoters, enhancers, class
switch regions, recombination signals and the like, comprise

corresponding sequences derived from the heterologous DNA.
Alternatively, such regulatory sequences may be incorporated
into the transgene from the same or a related species of the
non-human animal used in the invention. For example, human
immunoglobulin gene segments may be combined in a transgene
with a rodent immunoglobulin enhancer sequence for use in a
transgenic mouse.

In a method of the invention, a transgenic non-human
animal containing germline unrearranged light and heavy
immunoglobulin transgenes - that undergo VDJ joining during
D-cell differentiation - is contacted with an antigen to induce
production of a heterologous antibody in a secondary repertoire
B-cell. Such induction causes somatic mutation in the
rearranged heavy and/or light chain transgenes contained in
primary repertoire B-cells to produce a heterologous antibody
having high affinity and specificity for the antigen.

Such antibody producing B-cells may be immortalized by
transforming with a virus, or with an oncogene containing DNA
construct, or alternatively, immortalized by fusing with a
myeloma cell line to form antibody secreting hybridomas. In
each instance, clones having sufficient affinity and
specificity for a particular antigen are selected to provide a
source of monoclonal antibody having low immunogenicity in the
species from which the immunoglobulin gene segments of the
transgenes are derived.

Also included in the invention are vectors and methods
to disrupt the endogenous immunoglobulin loci in the non-human
animal to be used in the invention. Such vectors and methods
utilize a transgene, preferably positive-negative selection
vector, which is constructed such that it targets the
functional disruption of a class of gene segments encoding a
heavy and/or light immunoglobulin chain endogenous to the
non-human animal used in the invention. Such endogenous gene
segments include diversity, joining and constant region gene
segments. In this aspect of the invention, the
positive-negative selection vector is contacted with at least
one embryonic stem cell of a non-human animal after which cells
are selected wherein the positive-negative selection vector has

integrated into the genome of the non-human animal by way of
homologous recombination. After transplantation, the resultant
transgenic non-human animal is substantially incapable of
mounting an immunoglobulin-mediated immune response as a result

5   of homologous integration of the vector. Such immune deficient
non-human animals may thereafter be used for study of immune
deficiencies or used as the recipient of heterologous
immunoglobulin heavy and light chain transgenes.

The invention also includes methods for generating a

10 synthetic variable region gene segment repertoire to be used in
the transgenes of the invention. The method comprises
generating a population of immunoglobulin V segment DNAs
wherein each of the V segment DNAs encodes an immunoglobulin V
segment and contains at each end a cleavage recognition site of

15 a restriction endonuclease. The population of immunoglobulin V
segment DNAs is thereafter concatenated to form the synthetic
immunoglobulin V segment repertoire.

Another aspect of the invention includes transgenic
nonhuman animals that contain functionally rearranged

20 heterologous heavy and light chain immunoglobulin transgenes in
the germline of the transgenic animal. Such animals contain
primary repertoire B-cells that express such rearranged heavy
and light transgenes. Such B-cells are capable of undergoing
somatic mutation when contacted with an antigen to form a

25 heterologous antibody having high affinity and specificity for
the antigen.

The invention also includes transgenic animals
containing germ line cells having a heavy and light transgene
wherein one of the said transSgenes contains rearranged gene

30 segments with the other containing unrearranged gene segments.
In the preferred embodiments, the rearranged transgene is a
light chain immunoglobulin transgene and the unrearranged
transgene is a heavy chain immunoglobulin transgene.

The invention also includes methods for producing

35 heterologous antibodies in a transgenic animal containing
primary repertoire B-cells having rearranged heavy and light
heterologous immunoglobulin transgenes. Such transgenic
animals may be obtained from any of the aforementioned

transgenic animals. Thus, the transgenic animal containing
unrearranged heavy and light transgenes, the transgenic animal
containing rearranged heavy and light transgenes or the animal
containing one rearranged and one unrearranged transgene in the
5   germline of the animal, each contain primary repertoire B-cells
having rearranged, heterologous heavy and light immunoglobulin
transgenes. In the method of the invention, a desired first
heterologous antibody is produced which is capable of binding a
first antigen. The rearranged immunoglobulin heavy and light
10 transgenes in the primary repertoire B-cells of such animals
are known to produce primary repertoire antibodies having
sufficient affinity for a second known antigen. In this
method, the transgenic non-human animal is contacted,
sequentially or simultaneously, with the first and second
15 antigen to induce production of the first heterologous antibody
by somatic mutation of the rearranged transgenes. The
secondary repertoire B-cells so produced are then manipulated
as previously described to immortalize the production of the
desired monoclonal antibody capable of binding the first
20 antigen.

The present invention also includes plasmids, useful
in cloning large DNA fragments (e.g., immunoglobulin genomic
fragments), that have an origin of replication (ORI), a copy
control region (e.g., ROP, or the copy control region of
25 pACYC177, or others known to those skilled in the art), and a
cloning site. The plasmids also include a transcription
terminator (e.g., trpR or others known to those skilled in the
art) downstream of endogenous plasmid-derived promoters such as
that of the ampicillin resistance gene (amp^R). The
30 transcription termination is located upstream of the cloning
site so that transcripts originating at the promoter are
terminated upstream of the cloning site. In a preferred
embodiment, the cloning site is flanked by rare restriction
sites, which are sites consisting of seven, eight, or more
35 nucleotides, instead of the six or fewer nucleotides that make
up more common restriction sites; e.g., Not I, Sfi I, and
Pac I. Rare restriction sites also include sites that contain
nucleotide sequences occurring rarely in natural DNA sequences;

i.e., less frequently than about once in every 8,000-10,000 nucleotides.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 depicts the complementarity determining regions CDR1, CDR2 and CDR3 and framework regions FR1, FR2, FR3 and FR4 in unrearranged genomic DNA and mRNA expressed from a rearranged immunoglobulin heavy chain gene,

Fig. 2 depicts the human λ chain locus,

Fig. 3 depicts the human κ chain locus,

Fig. 4 depicts the human heavy chain locus,

Figs. 5 and 6 depict the strategy for generating a synthetic V segment repertoire.

Fig. 7 depicts the strategy for functional disruption of endogenous immunoglobulin loci.

Fig. 8 depicts the T-cell mediated secondary response leading to maturation of the B-cell.

Fig. 9 depicts somatic mutation and clonal expansion of B-cells in response to two different antigens.

Fig. 10 depicts a transgene construct containing a rearranged IgM gene ligated to a 25 kb fragment that contains human γ3 and γ1 constant regions followed by a 700 bp fragment containing the rat chain 3' enhancer sequence.

Fig. 11 is a restriction map of the human κ chain locus depicting the fragments to be used to form a light chain transgene by way of in vivo homologous recombination.

Fig. 12 depicts the construction of pGP1.

Fig. 13 depicts the construction of the polylinker contained in pGP1.

Fig. 14 depicts the fragments used to construct a human heavy chain transgene of the invention.

Fig. 15 depicts the construction of pHIG1 and pCON1.

Fig. 16 depicts the human Cγ1 fragments which are inserted into pRE3 (rat enhancer 3') to form pREG2.

Fig. 17 depicts the construction of pHIG3' and PCON.

Fig. 18 depicts the fragment containing human D region segments used in construction of the transgenes of the invention.

Fig. 19 depicts the construction of pHIG2 (D segment containing plasmid).

Fig. 20 depicts the fragments covering the human Jκ and human Cκ gene segments used in constructing a transgene of the invention.

Fig. 21 depicts the structure of pEμ.

Fig. 22 depicts the construction of pKapH.

Figs. 23A through 23D depict the construction of a positive-negative selection vector for functionally disrupting the endogenous heavy chain immunoglobulin locus of mouse.

Figs. 24A through 24C depict the construction of a positive-negative selection vector for functionally disrupting the endogenous immunoglobulin light chain loci in mouse.

Figs. 25 a through e depict the structure of a kappa light chain targeting vector.

Figs. 26 a through f depict the structure of a mouse heavy chain targeting vector.

Fig. 27 depicts the map of vector pGPe.

Fig. 28 depicts the structure of vector pJM2.

Fig. 29 depicts the structure of vector pCOR1.

Fig. 30 depicts the transgene constructs for pIGM1, pHC1 and pHC2.

Fig. 31 depicts the structure of pγe2.

Fig. 32 depicts the structure of pVGE1.

Fig. 33 depicts the assay results of human Ig expression in a pHC1 transgenic mouse.

Fig. 34 depicts the structure of pJCK1.

Fig. 35 depicts the construction of a synthetic heavy chain variable region.

Table 1 depicts the sequence of vector pGPe.

Table 2 depicts the sequence of gene $V_H49.8$.

DETAILED DESCRIPTION

The design of a transgenic non-human animal that responds to foreign antigen stimulation with a heterologous antibody repertoire, requires that the heterologous immunoglobulin transgenes contained within the transgenic animal function correctly throughout the pathway of B-cell development. Accordingly, the transgenes in one aspect of the

invention are constructed so as to produce one or all of the
following: (1) high level and cell-type specific expression,
(2) functional gene rearrangement, (3) activation of and
response to allelic exclusion, (4) expression of a sufficient
5   primary repertoire, (5) signal transduction, (6) class
switching, (7) somatic hypermutation, and (8) domination of the
transgene antibody locus during the immune response.

       As will be apparent from the following disclosure, not
all of the foregoing criteria need be met. For example, in
10   those embodiments wherein the endogenous immunoglobulin loci of
the transgenic animal are functionally disrupted, the transgene
need not activate allelic exclusion. Further, in those
embodiments wherein the transgene comprises a functionally
rearranged heavy and/or light chain immunoglobulin gene, the
15   second criteria of functional gene rearrangement is
unnecessary, at least for that transgene which is already
rearranged. For background on molecular immunology, see,
Fundamental Immunology, 2nd edition (1989), Paul William E.,
ed. Raven Press, N.Y.
20   The Structure and Generation of Antibodies
       Immunoglobulins, also known as antibodies, are a group
of glycoproteins present in the serum and tissue fluids of all
mammals. They are produced in large amounts by plasma cells
(also referred to herein as "secondary repertoire B-cells")
25   which develop from precursor B lymphocytes (referred to herein
as "primary repertoire B-cells"). Such primary repertoire
.B-cells carry membrane-bound immunoglobulin which is similar to
that produced by the fully differentiated secondary repertoire
B-cell. Contact between primary repertoire B-cells and foreign
30   antigen is required for the induction of antibody formation.
       The basic structure of all immunoglobulins is based
upon a unit consisting of two identical light polypeptide
chains and two identical heavy polypeptide chains linked
together by disulfide bonds. Each light chain comprises two
35   regions known as the variable light chain region and the
constant light chain region. Similarly, the immunoglobulin
heavy chain comprises two regions designated the variable heavy
chain region and the constant heavy chain region. The constant

13

region for the heavy or light chain is encoded by genomic
sequences referred to as heavy or light constant region gene
segments. The use of a particular heavy chain gene segment
defines the class of immunoglobulin. For example, in humans,
5   the μ constant region gene segments define the IgM class of
antibody whereas the use of a γ, γ2, γ3 or γ4 constant region
gene segment defines the IgG class of antibodies as well as the
IgG subclasses IgG1 through IgG4.

The variable regions of the heavy and light
10   immunoglobulin chains together contain the antigen binding
domain of the antibody. Because of the need for diversity in
this region of the antibody to permit binding to a wide range
of antigens, the DNA encoding the initial or primary repertoire
variable region comprises a number of different DNA segments
15   derived from families of specific variable region gene
segments. In the case of the light chain variable region, such
families comprise variable (V) gene segments and joining (J)
gene segments. Thus, the initial variable region of the light
chain is encoded by one V gene segment and one J gene segment
20   each selected from the family of V and J gene segments
contained in the genomic DNA of the organism. In the case of
the heavy chain variable region, the DNA encoding the initial
or primary repertoire variable region of the heavy chain
comprises one heavy chain V gene segment, one heavy chain
25   diversity (D) gene segment and one J gene segment, each
selected from the appropriate V, D and J families of
immunoglobulin gene segments in genomic DNA.

The Primary Repertoire

30   The process for generating DNA encoding the heavy and
light chain immunoglobulin genes occurs primarily in developing
B-cells. Prior to the joining of various immunoglobulin gene
segments, the V, D, J and constant (C) gene segments are found,
for the most part, in clusters of V, D, J and C gene segments
35   in the precursors of primary repertoire B-cells. Generally,
all of the gene segments for a heavy or light chain are located
in relatively close proximity on a single chromosome. Such
genomic DNA prior to recombination of the various

14

immunoglobulin gene segments is referred to herein as "unrearranged" genomic DNA. During B-cell differentiation, one of each of the appropriate family members of the V, D, J (or only V and J in the case of light chain genes) gene segments

5   are recombined to form functionally rearranged heavy and light immunoglobulin genes. Such functional rearrangement is of the variable region segments to form DNA encoding a functional variable region. This gene segment rearrangement process appears to be sequential. First, heavy chain D-to-J joints are

10 made, followed by heavy chain V-to-DJ joints and light chain V-to-J joints. The DNA encoding this initial form of a functional variable region in a light and/or heavy chain is referred to as "functionally rearranged DNA" or "rearranged DNA". In the case of the heavy chain, such DNA is referred to

15 as "rearranged heavy chain DNA" and in the case of the light chain, such DNA is referred to as "rearranged light chain DNA". Similar language is used to describe the functional rearrangement of the transgenes of the invention.

        The recombination of variable region gene segments to

20 form functional heavy and light chain variable regions is mediated by recombination signal sequences (RSS's) that flank recombinationally competent V, D and J segments. RSS's necessary and sufficient to direct recombination, comprise a dyad-symmetric heptamer, an AT-rich nonamer and an intervening

25 spacer region of either 12 or 23 base pairs. These signals are conserved among the different loci and species that carry out D-J (or V-J) recombination and are functionally interchangeable. See Oettinger, et al. (1990), Science, 248, 1517-1523 and references cited therein. The heptamer comprises

30 the sequence CACAGTG or its analogue followed by a spacer of unconserved sequence and then a nonamer having the sequence ACAAAAACC or its analogue. These sequences are found on the J, or downstream side, of each V and D gene segment. Immediately preceding the germline D and J segments are again two

35 recombination signal sequences, first the nonamer and then the heptamer again separated by an unconserved sequence. The heptameric and nonameric sequences following a V_L, V_H or D segment are complementary to those preceding the J_L, D or J_H

15

segments with which they recombine. The spacers between the
heptameric and nonameric sequences are either 12 base pairs
long or between 22 and 24 base pairs long.

5   In addition to the rearrangement of V, D and J
segments, further diversity is generated in the primary
repertoire of immunoglobulin heavy and light chain by way of
variable recombination between the V and J segments in the
light chain and between the D and J segments of the heavy
chain. Such variable recombination is generated by variation
10   in the exact place at which such segments are joined. Such
variation in the light chain typically occurs within the last
codon of the V gene segment and the first codon of the J
segment. Similar imprecision in joining occurs on the heavy
chain chromosome between the D and J_H segments and may extend
15   over as many as 10 nucleotides. Furthermore, several
nucleotides may be inserted between the D and J_H and between
the V_H and D gene segments which are not encoded by genomic
DNA. The addition of these nucleotides is known as N-region
diversity.

20   After VJ and/or VDJ rearrangement, transcription of
the rearranged variable region and one or more constant region
gene segments located downstream from the rearranged variable
region produces a primary RNA transcript which upon appropriate
RNA splicing results in an mRNA which encodes a full length
25   heavy or light immunoglobulin chain. Such heavy and light
chains include a leader signal sequence to effect secretion
through and/or insertion of the immunoglobulin into the
transmembrane region of the B-cell. The DNA encoding this
signal sequence is contained within the first exon of the V
30   segment used to form the variable region of the heavy or light
immunoglobulin chain. Appropriate regulatory sequences are
also present in the mRNA to control translation of the mRNA to
produce the encoded heavy and light immunoglobulin polypeptides
which upon proper association with each other form an antibody
35   molecule.

The net effect of such rearrangements in the variable
region gene segments and the variable recombination which may
occur during such joining, is the production of a primary

16

antibody repertoire. Generally, each B-cell which has
differentiated to this stage, produces a single primary
repertoire antibody. During this differentiation process,
cellular events occur which suppress the functional

5   rearrangement of gene segments other than those contained
within the functionally rearranged Ig gene. The process by
which diploid B-cells maintain such mono-specificity is termed
allelic exclusion.

10   The Secondary Repertoire
           B-cell clones expressing immunoglobulins from within
the set of sequences comprising the primary repertoire are
immediately available to respond to foreign antigens. Because
of the limited diversity generated by simple VJ and VDJ

15   joining, the antibodies produced by the so-called primary
response are of relatively low affinity. Two different types
of B-cells make up this initial response: precursors of primary
antibody-forming cells and precursors of secondary repertoire
B-cells (Linton, et al. (1989), Cell, 59, 1049-1059). The

20   first type of B-cell matures into IgM-secreting plasma cells in
response to certain antigens. The other B-cells respond to
initial exposure to antigen by entering a T-cell dependent
maturation pathway. It is during this T-cell dependent
maturation of B-cells that a second level of diversity is

25   generated by a process termed somatic mutation (sometimes also
referred to as hypermutation). These primary repertoire B-cells
use the immunoglobulin molecules on their surfaces to bind and
internalize the foreign antigen. If the foreign antigen is a
protein or is physically linked to another protein antigen,

30   that protein antigen is then processed and presented on the
cell surface by a major histocompatibility complex (MHC)
molecule to a helper T-cell which in turn induces maturation of
the B-cell. Lanzavecchia (1985), Nature, 314, 537. This
overall maturation of the B-cell is known as the secondary

35   response.
           During the T-cell dependent maturation of antigen
stimulated B-cell clones, the structure of the antibody
molecule on the cell surface changes in two ways: the constant

17

region switches to a non-IgM subtype and the sequence of the
variable region is modified by multiple single amino acid
substitutions to produce a higher affinity antibody molecule.
It is this process of somatic mutation, followed by the
5   selection of higher affinity clones, that generates highly
specific and tightly binding immunoglobulins characterized by
the Ig mediated immune response.

        As previously indicated, each variable region of a
heavy or light Ig chain contains an antigen binding domain. It
10   has been determined by amino acid and nucleic acid sequencing
that somatic mutation during the secondary response occurs
throughout the V region including the three complementary
determining regions (CDR1, CDR2 and CDR3) also referred to as
hypervariable regions 1, 2 and 3. The CDR1 and CDR2 are
15   located within the variable gene segment whereas the CDR3 is
largely the result of recombination between V and J gene
segments or V, D and J gene segments. Those portions of the
variable region which do not consist of CDR1, 2 or 3 are
commonly referred to as framework regions designated FR1, FR2,
20   FR3 and FR4. See Fig. 1. During hypermutation, the rearranged
DNA is mutated to give rise to new clones with altered Ig
molecules. Those clones with higher affinities for the foreign
antigen are selectively expanded by helper T-cells, giving rise
to affinity maturation of the expressed antibody. Clonal
25   selection typically results in expression of clones containing
new mutation within the CDR1, 2 and/or 3 regions. However,
mutations outside these regions also occur which influence the
specificity and affinity of the antigen binding domain.

30   Transgenic Non-Human Animals Capable
of Producing Heterologous Antibody

        Transgenic non-human animals in one aspect of the
invention are produced by introducing at least one of the
35   immunoglobulin transgenes of the invention (discussed
hereinafter) into a zygote or early embryo of a non-human
animal. The non-human animals which are used in the invention
generally comprise any mammal which is capable of rearranging
immunoglobulin gene segments to produce a primary antibody

18

response and, which, in addition, are capable of mounting a
secondary response by way of somatic mutation of such
rearranged Ig genes. A particularly preferred non-human animal
is the mouse or other members of the rodent family. Mice are

5   particularly useful since their immune system has been
extensively studied, including the genomic organization of the
mouse heavy and light immunoglobulin loci. See e.g.
Immunoglobulin Genes, Academic Press, T. Honjo, F.W. Alt and
T.H. Rabbitts, eds. (1989).

10   However, the invention is not limited to the use of
mice. Rather, any non-human mammal which is capable of
mounting a primary and secondary antibody response may be used.
Such animals include non-human primates, such as chimpanzee,
bovine, ovine and porcine species, other members of the rodent

15   family, e.g. rat, as well as rabbit and guinea pig. Particular
preferred animals are mouse, rat, rabbit and guinea pig, most
preferably mouse.

As used herein, the term "antibody" refers to a
glycoprotein comprising at least two identical light

20   polypeptide chains and two identical heavy polypeptide chains
linked together by disulfide bonds. Each of the heavy and
light polypeptide chains contains a variable region (generally
the amino terminal portion of the polypeptide chain) which
contains a binding domain which interacts with antigen. Each

25   of the heavy and light polypeptide chains also comprises a
constant region of the polypeptide chains (generally the
carboxyl terminal portion) some of which sequences mediate the
binding of the immunoglobulin to host tissues including various
cells of the immune system, some phagocytic cells and the first

30   component (Clq) of the classical complement system.

As used herein, a "heterologous antibody" is defined
in relation to the transgenic non-human organism producing such
an antibody. It is defined as an antibody having an amino acid
sequence or an encoding DNA sequence corresponding to that

35   found in an organism not consisting of the transgenic non-human
animal. Thus, prior to rearrangement of a transgene containing
various heavy or light chain gene segments, such gene segments
may be readily identified, e.g. by hybridization or DNA

sequencing, as being from a species of organism other than the transgenic animal. For example, in one embodiment of the invention, various gene segments from the human genome are used in heavy and light chain transgenes in an unrearranged form.

5   In this embodiment, such transgenes are introduced into mice. The unrearranged gene segments of the light and/or heavy chain transgene have DNA sequences unique to the human species which are distinguishable from the endogenous immunoglobulin gene segments in the mouse genome. They may be readily detected in

10 unrearranged form in the germ line and somatic cells not consisting of B-cells and in rearranged form in B-cells.

In an alternate embodiment of the invention, the transgenes comprise rearranged heavy and/or light immunoglobulin transgenes. Specific segments of such

15 transgenes corresponding to functionally rearranged VDJ or VJ segments, contain immunoglobulin DNA sequences which are also clearly distinguishable from the endogenous immunoglobulin gene segments in the mouse.

Such differences in DNA sequence are also reflected in

20 the amino acid sequence encoded by such human immunoglobulin transgenes as compared to those encoded by mouse B-cells. Thus, human immunoglobulin amino acid sequences may be detected in the transgenic non-human animals of the invention with antibodies specific for immunoglobulin epitopes encoded by

25 human immunoglobulin gene segments.

Transgenic B-cells containing unrearranged transgenes from human or other species functionally recombine the appropriate gene segments to form functionally rearranged light and heavy chain variable regions. It is to be understood that

30 the DNA of such rearranged transgenes for the most part will not correspond exactly to the DNA sequence of the gene segments from which such rearranged transgenes were obtained. This is due primarily to the variations introduced during variable recombination and because of mutations introduced by

35 hypermutation during the secondary response. Notwithstanding such modifications in DNA (as well as in amino acid) sequence, it will be readily apparent that the antibody encoded by such rearranged transgenes has a DNA and/or amino acid sequence

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which is heterologous to that normally encountered in the non-
human animal used to practice the invention.

The term "substantial identity", when referring to
polypeptides, indicates that the polypeptide or protein in
5   question exhibits at least about 30% identity with an entire
naturally occurring protein or a portion thereof, usually at
least about 70% identity, and preferably at least about 95%
identity. As used herein, the terms "isolated", "substantially
pure" and "substantially homogenous" are used interchangeably
10   herein and describe a polypeptide protein which has been
separated from components which naturally accompany it.
Typically, a monomeric protein is substantially pure when at
least about 60 to 75% of a sample exhibits a single polypeptide
backbone. Minor variants or chemical modifications typically
15   share the same polypeptide sequence. A substantially pure
protein will typically comprise over about 85 to 90% of a
protein sample, more usually about 95%, and preferably will be
over about 99% pure. Protein purity or homogeneity may be
indicated by a number of means well known in the art, such as
20   polyacrylamide gel electrophoresis of a protein sample,
followed by visualizing a single polypeptide band on a
polyacrylamide gel upon staining. For certain purposes high
resolution will be needed and HPLC or a similar means for
purification utilized. A polypeptide is substantially free of
25   naturally-associated components when it is separated from the
native contaminants which accompany it in its natural state.
Thus, a polypeptide which is synthesized in a cellular system
different from the cell from which it naturally originates will
be substantially free from its naturally-associated components.
30

Unrearranged Transgenes

As used herein, an "unrearranged immunoglobulin heavy
chain transgene" comprises DNA encoding at least one variable
gene segment, one diversity gene segment, one joining gene
35   segment and one constant region gene segment. Each of the gene
segments of said heavy chain transgene are derived from, or has
a sequence corresponding to, DNA encoding immunoglobulin heavy
chain gene segments from a species not consisting of the

non-human animal into which said transgene is introduced.
Similarly, as used herein, an "unrearranged immunoglobulin
light chain transgene" comprises DNA encoding at least one
variable gene segment, one joining gene segment and at least

5   one constant region gene segment wherein each gene segment of
said light chain transgene is derived from, or has a sequence
corresponding to, DNA encoding immunoglobulin light chain gene
segments from a species not consisting of the non-human animal
into which said light chain transgene is introduced.

10   Such heavy and light chain transgenes in this aspect
of the invention contain the above-identified gene segments in
an unrearranged form. Thus, interposed between the V, D and J
segments in the heavy chain transgene and between the V and J
segments on the light chain transgene are appropriate

15   recombination signal sequences (RSS's). In addition, such
transgenes also include appropriate RNA splicing signals to
join a constant region gene segment with the VJ or VDJ
rearranged variable region.

            To the extent that the heavy chain transgene contains

20   more than one C region gene segment, e.g. $C\mu$ and $C\gamma 1$ from the
human genome, as explained below "switch regions" are
incorporated upstream from each of the constant region gene
segments and downstream from the variable region gene segments
to permit recombination between such constant regions to allow

25   for immunoglobulin class switching, e.g. from IgM to IgG. Such
heavy and light immunoglobulin transgenes also contain
transcription control sequences including promoter regions
situated upstream from the variable region gene segments which
contain OCTA and TATA motifs.

30   In addition to promoters, other regulatory sequences
which function primarily in B-lineage cells are used. Thus,
for example, a light chain enhancer sequence situated
preferably between the J and constant region gene segments on
the light chain transgene is used to enhance transgene

35   expression, thereby facilitating allelic exclusion. In the
case of the heavy chain transgene, regulatory enhancers and
also employed.

Although the foregoing promoter and enhancer
regulatory control sequences have been generically described,
such regulatory sequences may be heterologous to the nonhuman
animal being derived from the genomic DNA from which the
5   heterologous transgene immunoglobulin gene segments are
obtained. Alternately, such regulatory gene segments are
derived from the corresponding regulatory sequences in the
genome of the non-human animal, or closely related species,
which contains the heavy and light transgene. Such regulatory
10   sequences are used to maximize the transcription and
translation of the transgene so as to induce allelic exclusion
and to provide relatively high levels of transgene expression.

In the preferred embodiments, each of the
immunoglobulin gene segments contained on the heavy and light
15   Ig transgenes are derived from, or have sequences corresponding
to, genomic DNA, cDNA or portions thereof from a species or
individual which is heterologous to the non-human animal into
which the transgene is to be introduced. As a consequence,
when such gene segments are functionally rearranged and
20   hypermutated in the transgenic non-human animal, the
heterologous antibody encoded by such heavy and light
transgenes will have an amino acid sequence and overall
secondary and terteriary structure which provides specific
utility against a desired antigen when used therapeutically in
25   the organism from which the Ig gene segments are derived. In
addition, such antibodies demonstrate substantially reduced
immunogenicity as compared to antibodies which are "foreign" to
the organism treated.

For example, in the preferred embodiments, gene
30   segments are derived from human beings. The transgenic
non-human animals harboring such heavy and light transgenes are
capable of mounting an Ig-mediated immune response to a
specific antigen administered to such an animal. B-cells are
produced within such an animal which are capable of producing
35   heterologous human antibody. After immortalization, and the
selection for an appropriate monoclonal antibody (Mab), e.g. a
hybridoma, a source of therapeutic human monoclonal antibody is

provided. Such human Mabs have significantly reduced
immunogenicity when therapeutically administered to humans.

Examples of antigens which may be used to generate
heterologous antibodies in the transgenic animals of the

5   invention containing human immunoglobulin transgenes include
bacterial, viral and tumor antigen as well as particular human
B- and T-cell antigens associated with graft rejection or
autoimmunity.

Although the preferred embodiments disclose the

10   construction of heavy and light transgenes containing human
gene segments, the invention is not so limited. In this
regard, it is to be understood that the teachings described
herein may be readily adapted to utilize immunoglobulin gene
segments from a species other than human beings. For example,

15   in addition to the therapeutic treatment of humans with the
antibodies of the invention, therapeutic antibodies encoded by
appropriate gene segments may be utilized to generate
monoclonal antibodies for use in the veterinary sciences. For
example, the treatment of livestock and domestic animals with

20   species-related monoclonal antibodies is also contemplated by
the invention. Such antibodies may be similarly generated by
using transgenes containing immunoglobulin gene segments from
species such as bovine, ovine, porcine, equine, canine, feline
and the like.

25

Class Switching

The use of μ or δ constant regions is largely
determined by alternate splicing, permitting IgM and IgD to be
coexpressed in a single cell. The other heavy chain isotypes

30   (γ, α, and ϵ) are only expressed natively after a gene
rearrangement event deletes the $C\mu$ and $C\delta$ exons. This gene
rearrangement process, termed class switching, occurs by
recombination between so called switch segments located
immediately upstream of each heavy chain gene (except δ). The

35   individual switch segments are between 2 and 10 kb in length,
and consist primarily of short repeated sequences. The exact
point of recombination differs for individual class switching
events.

24

        The ability of a transgene construction to switch
isotypes during B-cell maturation has not been directly tested
in transgenic mice; however, transgenes should carry out this
function. Durdik et al. (1989) <u>Proc. Natl. Acad. Sci. USA</u>, <u>86</u>,
5   2346-2350) microinjected a rearranged mouse μ heavy chain gene
construct and found that in four independent mouse lines, a
high proportion of the transgenic B-cells expressed the
transgene-encoded variable region associated with IgG rather
than IgM. Thus, isotype switching appears to have taken place
10   between the transgene and the endogenous γ constant region on
another chromosome.

        As used herein, the term switch sequence thus refers
to those DNA sequences responsible for switch recombination. A
"switch donor" sequence, typically a μ switch region, will be
15   5' (i.e., upstream) of the construct region to be deleted
during the switch recombination. The "switch acceptor" region
will be between the construct region to be deleted and the
replacement constant region (e.g., γ, ϵ, etc.). As there is no
specific site where recombination always occurs, the final gene
20   sequence will typically not be predictable from the construct.

        The switch (S) region of the μ gene, S_μ, is located
about 1 to 2 kb 5' to the coding sequence and is composed of
numerous tandem repeats of sequences of the form
$(GAGCT)_n(GGGGT)$, where n is usually 2 to 5 but can range as
25   high as 17. (<u>See</u> T. Nikaido, et al. (1981): <u>Nature</u>, <u>292</u>:845-
848.)

        Similar internally repetitive switch sequences
spanning several kilobases have been found 5' of the other C_H
genes. The Sα region has been sequenced and found to consist
30   of tandemly repeated 80-bp homology units, whereas $S_{\gamma 2a}$, $S_{\gamma 2b}$,
and $S_{\gamma 3}$ all contain repeated 49-bp homology units very similar
to each other. (<u>See</u>, P. Szurek, et al. (1985): <u>J. Immunol</u>,
<u>135</u>:620-626 and T. Nikaido, et al. (1982): <u>J. Biol. Chem.</u>,
<u>257</u>:7322-7329.) All the sequenced S regions include numerous
35   occurrences of the pentamers GAGCT and GGGGT that are the basic
repeated elements of the S_μ gene (T. Nikaido, et al. (1982): <u>J.
Biol. Chem.</u>, <u>257</u>:7322-7329); in the other S regions these

25

pentamers are not precisely tandemly repeated as in S_μ, but
instead are embedded in larger repeat units.

    The $S_{\gamma 1}$ region has an additional higher-order
structure: two direct repeat sequences flank each of two

5   clusters of 49-bp tandem repeats. (See M. R. Mowatt, et al
(1986): J. Immunol., 136:2674-2683). Switch regions of human H
chain genes have been found very similar to their mouse
homologs. Generally, unlike the enzymatic machinery of V-J
recombination, the switch machinery can apparently accommodate

10   different alignments of the repeated homologous regions of
germline S precursors and then join the sequences at different
positions within the alignment. (See, T. H. Rabbits, et al.
(1981): Nucleic Acids Res., 9:4509-4524 and J. Ravetch, et al.
(1980): Proc. Natl. Acad. Sci. USA, 77:6734-6738.)

15   Induction of class switching appears to be associated
with sterile transcripts that initiate upstream of the switch
segments (Lutzker et al., 1988 Mol. Cell. Biol., 8, 1849;
Stavnezer et al. 1988 Proc. Natl. Acad. Sci. USA, 85, 7704;
Esser and Radbruch 1989 EMBO J., 8, 483; Berton et al. 1989

20   Proc. Natl. Acad. Sci. USA, 86, 2829; Rothman et al. 1990 Int.
Immunol. 2, 621). For example, the observed induction of the
$\gamma 1$ sterile transcript by IL-4 and inhibition by IFN-γ
correlates with the observation that IL-4 promotes class
switching to $\gamma 1$ in B-cells in culture, while IFN-γ inhibits $\gamma 1$

25   expression. Ideally then, transgene constructs that are
intended to undergo class switching should include all of the
cis-acting sequences necessary to regulate these sterile
transcripts. An alternative method for obtaining class
switching in transgenic mice ($\sigma\mu$ and $\epsilon\mu$) involves the inclusion

30   of the 400 bp direct repeat sequences that flank the human μ
gene (Yasui et al. 1989 Eur. J. Immunol., 19, 1399).
Homologous recombination between these two sequences deletes
the μ gene in IgD-only B-cells.

35   <u>Monoclonal Antibodies</u>
        Monoclonal antibodies can be obtained by various
techniques familiar to those skilled in the art. Briefly,
spleen cells from an animal immunized with a desired antigen

26

are immortalized, commonly by fusion with a myeloma cell (see, Kohler and Milstein, Eur. J. Immunol., 6:511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other

5   methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the

10   peritoneal cavity of a vertebrate host. Various techniques useful in these arts are discussed, for example, in Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York (1988) including: immunization of animals to produce immunoglobulins; production of monoclonal antibodies; labeling

15   immunoglobulins for use as probes; immunoaffinity purification; and immunoassays.

                  The Transgenic Primary Repertoire
A.   The Human Immunoglobulin Loci

20   An important requirement for transgene function is the generation of a primary antibody repertoire that is diverse enough to trigger a secondary immune response for a wide range of antigens. The size of the human immunoglobulin loci encoding the various gene segments for heavy and light chains

25   is quite large. For example, in the human genome the three separate loci for the λ light chain locus, the κ light chain locus and the heavy chain locus together occupy over 5 Mb of DNA or almost 0.2% of the entire genome. Each locus consists of multiple variable segments that recombine during B-cell

30   development with a joining region segment (and, the heavy chain locus with diversity region segments) to form complete V region exons. Such rearranged light chain genes consist of three exons: a signal peptide exon, a variable region exon and a constant region exon. The rearranged heavy chain gene is

35   somewhat more complex. It consists of a signal peptide exon, a variable region exon and a tandem array of multi-domain constant region regions, each of which is encoded by several exons. Each of the constant region genes encode the constant

portion of a different class of immunoglobulins. During B-cell development, V region proximal constant regions are deleted leading to the expression of new heavy chain classes. For each heavy chain class, alternative patterns of RNA, splicing give

5   rise to both transmembrane and secreted immunoglobulins.

Approximately 40% of human serum antibody molecules contain λ light chains. The structure of this locus, which maps to chromosome 22, is the least well characterized (Fig. 2). It consists of an unknown number of V segments upstream of

10   a tandem array of six constant region genes, each of which is linked to a single J segment. In addition, two more constant region segments with associated J segments have been isolated, although their linkage with the rest of the λ cluster has not been established, and it is not known if they are used. E.

15   Selsing, et al., "Immunoglobulin Genes", Academic Press, T. Honjo, F.W. Alt and T.H. Rabbitts, eds. (1989).

The κ light chain locus is spread out over three clusters on chromosome 2 (Fig. 3). The first two clusters, covering 850 and 250 kb respectively contain only variable

20   region gene segments. The third cluster, covering about 1 Mb, contains approximately 40 V gene segments upstream of a cluster of 5 J segments followed by a single constant region gene segment. A total of 84 V gene segments have been identified, and approximately half of these are thought to be pseudogenes

25   (Zachau (1989) in Immunoglobulin Genes, Academic Press, T. Honjo, F.W. Alt, and T.H. Rabbitts, eds. pp. 91-110). Approximately 25 kb downstream of the CK region there is a "k deleting element" (κde). The κde sequence recombines with upstream sequences, causing the deletion of the κ constant

30   region in λ light chain expressing B-cells. This leads to isotopic exclusion in cells that successfully rearrange both κ and λ genes.

The human heavy chain locus is the largest and most diverse. It consists of approximately 200 V gene segments

35   spanning 2 Mb, approximately 30 D gene segments spanning about 40 kb, six J segments clustered within a 3 kb span, and nine constant region gene segments spread out over approximately 300 kb. The entire locus spans approximately 2.5 Mb of the distal

portion of the long arm of chromosome 14 (Fig. 4). The heavy
chain V segments can be grouped into six families on the basis
of sequence similarity. There are approximately 60 members of
the V_H1 family, 30 V_H2 segments, 80 V_H3 segments, 30 V_H4

5   segments, three V_H5 segments, and one V_H6 segment. Berman,
J.E., et al. (1988), EMBO J., 7, 727-738. In the human heavy
chain locus, the members of individual V families are
intermingled, unlike the mouse locus where related V segments
are clustered. The single member of the VH6 family is the most

10   proximal of the V segments, mapping to within 90 kb of the
constant region gene segments. Sato, T., et al. (1988),
Biochem. Biophys. Res. Comm., 154, 265-271. All of the
functional D and J segments appear to lie in this 90 kb region
(Siebenlist, et al. (1981), Nature, 294, 631-635; Matsuda, et

15   al. (1988), EMBO J., 7, 1047-1051; Buluwela, et al. (1988),
EMBO J., 7, 2003-2010; Ichihara, et al. (1988), EMBO J., 7,
4141-4150; Berman, et al. (1988), EMBO J., 7, 727-738).

B.   Gene Fragment Transgenes

20   ### 1.   Heavy Chain Transgene

In a preferred embodiment, immunoglobulin heavy and
light chain transgenes comprise unrearranged genomic DNA from
humans. In the case of the heavy chain, a preferred transgene
comprises a NotI fragment having a length between 670 to 830

25   kb. The length of this fragment is ambiguous because the 3'
restriction site has not been accurately mapped. It is known,
however, to reside between the α1 and ψα gene segments (see
Fig. 4). This fragment contains members of all six of the
known V_H families, the D and J gene segments, as well as the μ,

30   δ, γ3, γ1 and α1 constant regions. Berman, et al. (1988), EMBO
J., 7, 727-738. A transgenic mouse line containing this
transgene correctly expresses all of the heavy chain classes
required for B-cell development as well as a large enough
repertoire of variable regions to trigger a secondary response

35   for most antigens.

2. <u>Light Chain Transgene</u>

A genomic fragment containing all of the necessary
gene segments and regulatory sequences from a human light chain
locus may be similarly constructed. Such a construct is
5   described in the Examples.

C.   Transgenes Generated Intracellularly
     <u>by In Vivo Recombination</u>

10   It is not necessary to isolate the all or part of the
heavy chain locus on a single DNA fragment. Thus, for example,
the 670-830 kb NotI fragment from the human immunoglobulin
heavy chain locus may be formed <u>in vivo</u> in the non-human animal
during transgenesis. Such <u>in vivo</u> transgene construction is
15   produced by introducing two or more overlapping DNA fragments
into an embryonic nucleus of the non-human animal. The
overlapping portions of the DNA fragments have DNA sequences
which are substantially homologous. Upon exposure to the
recombinases contained within the embryonic nucleus, the
20   overlapping DNA fragments homologously recombined in proper
orientation to form the 670-830 kb NotI heavy chain fragment.

It is to be understood, however, that <u>in vivo</u>
transgene construction can be used to form any number of
immunoglobulin transgenes which because of their size are
25   otherwise difficult, or impossible, to make or manipulate by
present technology. Thus, <u>in vivo</u> transgene construction is
useful to generate immunoglobulin transgenes which are larger
than DNA fragments which may be manipulated by YAC vectors
(Murray and Szostak (1983), <u>Nature</u>, <u>305</u>, 189-193). Such <u>in</u>
30   <u>vivo</u> transgene construction may be used to introduce into a
non-human animal substantially the entire immunoglobulin loci
from a species not consisting of the transgenic non-human
animal. Thus, although several groups have successfully
constructed libraries containing 50-200 kb of DNA fragments in
35   YAC vectors (Burke, et al. (1987), <u>Science</u>, <u>236</u>, 806-812;
Traver, et al. (1989), <u>Proc. Natl. Acad. Sci. USA</u>, <u>86</u>, 5898--
5902) and used polyamine condensation to produce YAC libraries
ranging in size from 200 to approximately 1000 kb (McCormick,
et al. (1989), <u>Proc. Natl. Acad. Sci USA</u>, <u>86</u>, 9991-9995),

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multiple overlapping fragments covering substantially more than
the 670-830 kb NotI fragment of the human constant region
immunoglobulin loci are expected to readily produce larger
transgenes by the methods disclosed herein.

5   In addition to forming genomic immunoglobulin
transgenes, in vivo homologous recombination may also be
utilized to form "mini-locus" transgenes as described in the
Examples.

          In the preferred embodiments utilizing in vivo
10   transgene construction, each overlapping DNA fragment
preferably has an overlapping substantially homologous DNA
sequence between the end portion of one DNA fragment and the
end portion of a second DNA fragment. Such overlapping
portions of the DNA fragments preferably comprise about 500 bp
15   to about 2000 bp, most preferably 1.0 kb to 2.0 kb. Homologous
recombination of overlapping DNA fragments to form transgenes
in vivo is further described in commonly assigned U.S. Patent
Application entitled "Intracellular Generation of DNA by
Homologous Recombination of DNA Fragments" filed August 29,
20   1990, under U.S.S.N. 07/574,747.

     D.   Minilocus Transgenes
          As used herein, the term "immunoglobulin minilocus"
refers to a DNA sequence (which may be within a longer
25   sequence), usually of less than about 150 kb, typically between
about 25 and 100 kb, containing at least one each of the
following: a functional variable (V) gene segment, a
functional joining (J) region segment, a functional constant
(C) region gene segment, and--if it is a heavy chain minilocus-
30   -a functional diversity (D) region segment, such that said DNA
sequence contains at least one substantial discontinuity (e.g.,
a deletion, usually of at least about 2 to 5 kb, preferably 10-
25 kb or more, relative to the homologous genomic DNA
sequence). A light chain minilocus transgene will be at least
35   25 kb in length, typically 50 to 60 kb. A heavy chain
transgene will typically be about 70 to 80 kb in length,
preferably at least about 60 kb with two constant regions
operably linked to switch regions, versus at least about 30 kb

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with a single constant region and incomplete switch regions.
Furthermore, the individual elements of the minilocus are
preferably in the germline configuration and capable of
undergoing gene rearrangement in the pre-B cell of a transgenic
5   animal so as to express functional antibody molecules with
diverse antigen specificities encoded entirely by the elements
of the minilocus.

In an alternate preferred embodiment, immunoglobulin
heavy and light chain transgenes comprise one or more of each
10   of the V, D, J and C gene segments. At least one of each
appropriate type gene segment is incorporated into the
minilocus transgene. With regard to the C segments for the
heavy chain transgene, it is preferred that the transgene
contain at least one μ gene segment and at least one other
15   constant region gene segment, more preferably a γ gene segment,
and most preferably $\gamma3$ or $\gamma1$. This preference is to allow for
class switching between IgM and IgG forms of the encoded
immunoglobulin to provide for somatic mutation and the
production of a secretable form of high affinity non-IgM
20   immunoglobulin. Other constant region gene segments may also
be used such as those which encode for the production of IgD,
IgA and IgE.

The heavy chain J region segments in the human
comprise six functional J segments and three pseudo genes
25   clustered in a 3 kb stretch of DNA. Given its relatively
compact size and the ability to isolate these segments together
with the μ gene and the 5' portion of the δ gene on a single 23
kb SFiI/SpeI fragment (Sado, et al. (1988), Biochem. Bioshys.
Res. Comm., 154, 264271), it is preferred that all of the J
30   region gene segments be used in the mini-locus construct.
Since this fragment spans the region between the μ and δ genes,
it is likely to contain all of the 3' cis-linked regulatory
elements required for μ expression. Furthermore, because this
fragment includes the entire J region, it contains the heavy
35   chain enhancer and the μ switch region (Mills, et al. (1983),
Nature, 306, 809; Yancopoulos and Alt (1986), Ann. Rev.
Immunol., 4, 339-368). It also contains the transcription
start sites which trigger VDJ joining to form primary

32

repertoire B-cells (Yancopoulos and Alt (1985), <u>Cell</u>, <u>40</u>, 271-281). Alternatively, a 36 kb BssHII/SpeI1 fragment, which includes part on the D region, may be used in place of the 23 kb SfiI/SpeI1 fragment. The use of such a fragment increases

5   the amount of 5' flanking sequence to facilitate efficient D-to-J joining.

The human D region consists of 4 or 5 homologous 9 kb subregions, linked in tandem (Siebenlist, et al. (1981), <u>Nature</u>, <u>294</u>, 631-635). Each subregion contains up to 10

10   individual D segments. Some of these segments have been mapped and are shown in Fig. 4. Two different strategies are used to generate a mini-locus D region. The first strategy involves using only those D segments located in a short contiguous stretch of DNA that includes one or two of the repeated D

15   subregions. A candidate is a single 15 kb fragment that contains 12 individual D segments. This piece of DNA consists of 2 contiguous EcoRI fragments and has been completely sequenced (Ichihara, et al. (1988), <u>EMBO J.</u>, <u>7</u>, 4141-4150). Twelve D segments should be sufficient for a primary

20   repertoire. However, given the dispersed nature of the D region, an alternative strategy is to ligate together several non-contiguous D-segment containing fragments, to produce a smaller piece of DNA with a greater number of segments.

At least one, and preferably more than one V gene

25   segment is used to construct the heavy chain minilocus transgene. A 10-15 kb piece of DNA containing one or two unrearranged V segments together with flanking sequences is isolated. A clone containing such DNA is selected using a probe generated from unique 5' sequences determined from the

30   transcribed V region of a characterized human hybridoma such as that which produces anti-cytomegalovirus antibody (Newkirk <u>et al</u>. (1988) <u>J. Clin. Invest.</u>, <u>81</u>, 1511-1518). The 5' untranslated sequence of the heavy chain mRNA is used to construct a unique nucleotide probe (preferably about 40

35   nucleotides in length) for isolating the original germline V segment that generated this antibody. Using a V segment that is known to be incorporated in an antibody against a known antigen not only insures that this V segment is functional, but

aids in the analysis of transgene participation in secondary
immune responses. This V segment is fused with the minilocus D
region and constant region fragments, discussed previously, to
produce a mini-locus heavy chain transgene.

5   Alternatively, a large, contiguous stretch of DNA
containing multiple V region segments is isolated from a YAC
library. Different sized pieces of DNA, containing different
numbers of V region segments, are tested for their ability to
provide a human antibody repertoire in the minilocus transgene
10   construct. It is also possible to build one large fragment
from several non-contiguous V segment containing fragments
using YAC vectors (Murray and Szostak (1983), _Nature_, _305_,
189-193), F factor-based plasmids (O'Conner, et al. (1989),
Science, _244_, 1307-1312) or the aforementioned _in vivo_
15   construction using recombination of overlapping fragments.
Alternatively, a synthetic V region repertoire (described
hereinafter) may be used.

A minilocus light chain transgene may be similarly
constructed from the human λ or κ immunoglobulin locus.
20   Construction of a κ light chain mini-locus is very similar to
construction of the heavy chain mini-locus, except that it is
much simpler because of its smaller size and lower complexity.
The human κ locus contains only one constant region segment;
and this segment, together with 5' and 3' enhancers, and all 5
25   of the functional J segments, can be isolated on a single 10 kb
DNA fragment. This fragment is co-injected together with a
minilocus V region constructed as described for the heavy chain
minilocus.

Thus, for example, an immunoglobulin heavy chain
30   minilocus transgene construct, e.g., of about 75 kb, encoding
V, D, J and constant region sequences can be formed from a
plurality of DNA fragments, at least two, three or four of
which each are either a V region sequence, a D region sequence,
a J and constant region sequence, a D and J and constant region
35   sequence or a constant region sequence, with each sequence
being substantially homologous to human gene sequences.
Preferably, the sequences are operably linked to transcription
regulatory sequences and are capable of undergoing

rearrangement. With two or more appropriately placed constant
region sequences (e.g., μ and γ) and switch regions, switch
recombination also occurs. An exemplary light chain transgene
construct similarly formed from a plurality of DNA fragments,
5   substantially homologous to human DNA and capable of undergoing
rearrangement will include at least two, three or four DNA
fragments, encoding V, D and constant regions, each fragment
comprising either a V region sequence, J and constant region
sequence or a constant region sequence.

10

E.   Methods for Determining Functional
     V Gene Segments and for Generating
     Synthetic V Segment Repertoire

15   Of the various families of gene segments, i.e., V, D,
J and C region gene segments, the number of V gene segments
generally far surpasses the number of corresponding gene
segments for the D, J and C region gene segments. By analogy
to the rabbit system wherein a single V gene segments is
20   utilized by approximately 90% of the antibodies produced
(Knight and Becker (1990), Cell, 60, 963-970), it is possible
to produce heavy and light transgenes containing a limited
number of V region gene segments, and as few as one V region
gene segments. Therefore, it is desirable to have a method to
25   determine which V region gene segments are utilized by a
particular organism, such as the human being, when mounting an
immunoglobulin-mediated immune response. According to this
approach, a single V gene segment when combining with the J or
DJ gene segments is capable of providing sufficient diversity
30   at CDR3 for the generation of a primary repertoire which upon
somatic mutation is able to provide further diversity
throughout the variable region, e.g. at CDR1 and CDR2 for the
production of high affinity antibodies.
             In this aspect of the invention, methods and vectors
35   are provided for determining which V gene segments are commonly
utilized by an organism during an immune response. This method
is based on determining which V segments are found in cDNA
synthesized from B-cell polyA+ RNA. Such methods and vectors
may also be used to facilitate the construction of a synthetic
40   V segment repertoire.

The outline of this strategy for identifying heavy
chain V segments and for generating a synthetic V segment
repertoire is depicted in Fig.s 5 and 6. It is similarly
applicable for identifying light chain V segments with

5   appropriate modification. The first step is the construction
of a cloning vector. The preferred starting material is a DNA
fragment (approximately 2 kb) containing an unrearranged V
segment together with 5' and 3' flanking sequences. This
fragment is cloned into a plasmid such as pGP1 or pGP2

10 described hereinafter containing a polylinker site flanked by
the rare cutting restriction sites designated "w" and "z" in
the Figs. 5 and 6 (the polylinkers and restriction sites of
pGP1 and pGP2 are described in the Examples). Oligonucleotide
directed mutagenesis is then used to introduce two new

15 restriction sites, "x" and "y" (generally each about 6
nucleotides in length). Restriction site "x" is placed
approximately 20 nucleotides from the 3' end of the intron
between the signal and V segment exon. Restriction site "y" is
placed approximately 20 nucleotides 3' of the V segment

20 junction, within the 23 bp spacer between the heptamer and
nonomer recombination signal sequences. Cutting the resulting
plasmid with enzymes "x" and "y" removes the second exon (V
segment), leaving the 5' flanking sequences, the V region
promoter, the signal peptide exon, the intron, a gap flanked by

25 "x" and "y" ends, the outside half of the recombination signal
sequence, and the 3' flanking sequences. This plasmid is
called pVH1.

The second step is the synthesis of four sets of
oligonucleotide primers, P1 through P4. P1 and P2 are

30 non-unique oligomers having approximately 50 nucleotides each
which are used to prime double stranded cDNA synthesis. P1
starts (going 5' to 3') with about 20 nucleotides of sequence
homologous to the antisense strand of the recombination signal
sequence in pVH1 (including the recognition sequence of

35 restriction enzyme "y"), and continues with approximately 30
nucleotides of antisense sequence hybridizing with about the
last 30 nucleotides of the VH framework region 3 (FR3). Random
bases are incorporated over about the last 30 nucleotides so as

to generate a set of primers that hybridize with all of the
different VH families. The second oligonucleotide, P2, is in
the sense orientation, and is homologous to the approximately
50 nucleotides beginning with the restriction site "x" in pVH1.

5   This includes the "x" restriction site, about the last 20
nucleotides of the intron, and about the first 30 nucleotides
of FR1. Again, about the last 30 nucleotides are non-unique so
as to accommodate different VH region segments.
Oligonucleotides P3 and P4 are homologous to about the first 20

10   nucleotides of P1 and P2 respectively. These oligos are unique
so as to avoid introducing new mutations into the V segments
and are used to amplify double stranded cDNA by way of the
polymerase chain reaction (PCR).

The 3' terminal portions of primers P1 and P2 which

15   are capable of hybridizing to and priming the synthesis of the
variable segments of the heavy or light immunoglobulin locus
may be readily determined by one skilled in the art. For
example, the nucleotide sequence for a number of human VH
genes have been published, see e.g. Berman, J.E., et al.

20   (1988), EMBO J., 7, 727-738 and Kabat, E.A., et al. (1987),
Sequences of Protein of Immunological interests, U.S. Dept.
Health & Human Services, Washington, D.C. Similarly, when used
to identify and/or generate V segments of the human light
immunoglobulin locus, the appropriate 3' sequence portions of

25   primers P1 and P2 may readily be determined from published
sequences. See e.g Kabat, E.A., et al., supra. In general,
those nucleotide positions which are conserved amongst various
V segments are also conserved in the 3' portion of the P1 and
P2 primers. For those nucleotide positions wherein variation

30   is observed amongst variable segments, such nucleotide
positions in the corresponding P1 and P2 primers are similarly
varied to provide P1 and P2 primers which comprise a pool of
primers which are capable of hybridizing to different VH or VL
segments.

35   The next step is to use these oligonucleotide primers
to generate a library of human heavy-chain V-region cDNA
sequences in the vector pHV1. P1 is used to prime first strand
cDNA synthesis from human B-cell polyA+ RNA. The RNA is base

hydrolyzed, and second strand synthesis primed with P2. Full
length, double stranded cDNA is then purified on an acrylamide
gel, electroeluted, and used as template for polymerase chain
reaction (PCR) amplification using oligonucleotide primers P3

5   and P4. Alternatively, cDNA is first synthesized by
conventional methods and this cDNA is used as a template for
the P1 primed reactor. The amplified product (approximately
0.3 kb) is then gel purified, cleaved with restriction enzymes
"x" and "y", and cloned into pHV1.

10   The resulting cDNA library represents a synthetic
genomic library of variable region segments and offers three
advantages over a conventional genomic library of variable
segments. First, this library contains no pseudogenes, while a
conventional library would contain up to 50% pseudogene

15 sequences. Second, the synthetic library is more compact than
a conventional library, containing one functional V segment per
2 kb of DNA, as opposed to one functional segment per 20 kb.
Finally, this approach leaves the V segment promoter sequences
accessible to manipulation.

20   Such a cDNA library may be biased towards particular
germline V segments because of differential expression. The
two sources of bias are: (i) differential rates of V segment
recombination, and (ii) differential selection of V segment
expressing B-cell clones. The first source of bias is dealt

25 with in two ways. First, fetal tissue is avoided as a source
of B-cell RNA, as the bias is most pronounced in the fetal
immunoglobulin repertoire. Second, the semi-random primers, P1
and P2, are divided into pools, each of which selectively
cross-hybridizes with different V segment families. These

30 primers are then used to generate 4 to 6 separate libraries,
thus insuring that all of the V region families are
represented. The second source of bias, differential selection
of B-cell clones, is also dealt with in two analogous ways.
First, a source of RNA that includes the minimum fraction of

35 antigen selected B-cells is used. Lymph nodes and spleen are
avoided. Adult bone marrow is one source of unselected
B-cells. However, it may contain a high proportion of
transcribed pseudogene sequences from pre-B-cells. Another

source of RNA is whole blood. Ninety percent of circulating
B-cells are immature μ or μ, δ expressing cells, and are recent
bone marrow immigrants. However, the level of antigen selected
IgG expressing cells can vary depending on the immune state of
5   the individual. Therefore, isolated polyA+ RNA is checked for
selected B-cell sequences by northern blot hybridization with γ
specific probes. If it is more practical to use spleen RNA,
and if this RNA contains a high fraction of IgG sequences, a
second approach is used to minimize selection bias. The first
10   strand of cDNA synthesis is primed with about a 40 nucleotide
constant-region exon 2 primer that is specific for IgM
transcripts. Second strand syntheses is then primed with P2,
and a third round of synthesis primed with P1. The cDNA from
this third round of synthesis provides the template for PCR
15   amplification using P3 and P4.

Once the variable region library has been generated,
the V segments used therein may by identified by standard
techniques, e.g. by way of sequencing and/or hybridization with
family specific or segment specific oligonucleotides as well as
20   differential amplification by PCR methods. Such
characterization of the V segment library provides information
as to the frequency and distribution of V segment utilization
in a particular organism and as a consequence, the
identification of V segments which may be used in the
25   construction of the various transgenes of the invention. Thus,
one or more predominant V gene segments may be used in the
above described mini-locus transgene construct. Further,
selected clones from such a library may be used to identify
genomic fragments containing frequently used V segments to
30   facilitate identification of genomic fragments containing a
particular desired V segment.

In addition, a synthetic V segment repertoire may be
constructed by concatenation of the library sequences. Large
repeating transgene tandem arrays, containing hundreds of
35   copies of the injected sequence, are commonly generated in the
production of transgenic mice. These tandem arrays are usually
quite stable. However, to ensure the stability of the
synthetic V region, blocks of random DNA between each 2 kb V

region segment are preferably introduced. These blocks of
random DNA are prepared by digesting and then religating
genomic DNA, so as to prevent the insertion of dominant
regulatory elements. Genomic DNA is preferably digested with
5   four frequent cutting restriction enzymes: AluI, DpnI, HaeIII,
and RsaI. This digest produces blunt ended fragments with an
average length of 64 nucleotides. Fragments in the size range
of 50 to 100 nucleotides are eluted from an acrylamide gel, and
religated. The relegated DNA is partially digested with MboI
10   and size fractionated. Fragments in the range of 0.5 to 2 kb
are cloned into the BamHI or BglII site of the polylinker of
the vector used to generate pVH1.

         The random sequence library is combined with the
synthetic V segment library to create a synthetic V segment
15   repertoire. Inserts from the random sequence library are
released with the enzymes "w" and "z", and purified away from
vector sequences. Inserts from the synthetic V segment library
are isolated by cutting with "w" and "z". Before purifying the
V segment inserts, this DNA is treated with calf-intestinal
20   phosphatase, to prevent self ligation. The V segment inserts
are then ligated together with the random inserts to generate
an alternating tandem array comprising a synthetic V segment
repertoire. This ligation mixture is size selected on a
sucrose gradient, and the 50-100 kb fraction microinjected
25   together with, for example, a D-J-constant mini-locus
construct. By directly injecting the synthetic V segment
repertoire without an intervening cloning step, it is possible
to take advantage of the fact that tandem arrays of injected
fragments become inserted at a single site. In this case such
30   tandem arrays are not completely redundant but lead to further
diversity. Alternatively, the synthetic V segment repertoire
may be combined with a D-J-C minilocus to form a heavy chain
transgene.

         A synthetic light chain immunoglobulin segment
35   repertoire may be similarly constructed using appropriate
primers for the light chain locus.

40

Functional Disruption of
Endogenous Immunoglobulin Loci

          The expression of successfully rearranged
5   immunoglobulin heavy and light transgenes is expected to have a
     dominant effect by suppressing the rearrangement of the
     endogenous immunoglobulin genes in the transgenic nonhuman
     animal. However, another way to generate a nonhuman that is
     devoid of endogenous antibodies is by mutating the endogenous
10   immunoglobulin loci. Using embryonic stem cell technology and
     homologous recombination, the endogenous immunoglobulin
     repertoire can be readily eliminated. The following describes
     the functional description of the mouse immunoglobulin loci.
     The vectors and methods disclosed, however, can be readily
15   adapted for use in other non-human animals.

          Briefly, this technology involves the inactivation of
     a gene, by homologous recombination, in a pluripotent cell line
     that is capable of differentiating into germ cell tissue. A
     DNA construct that contains an altered, copy of a mouse
20   immunoglobulin gene is introduced into the nuclei of embryonic
     stem cells. In a portion of the cells, the introduced DNA
     recombines with the endogenous copy of the mouse gene,
     replacing it with the altered copy. Cells containing the newly
     engineered genetic lesion are injected into a host mouse
25   embryo, which is reimplanted into a recipient female. Some of
     these embryos develop into chimeric mice that possess germ
     cells entirely derived from the mutant cell line. Therefore,
     by breeding the chimeric mice it is possible to obtain a new
     line of mice containing the introduced genetic lesion (reviewed
30   by Capecchi (1989), Science, 244, 1288-1292).

          Because the mouse λ locus contributes to only 5% of
     the immunoglobulins, inactivation of the heavy chain and/or
     κ-light chain loci is sufficient. There are three ways to
     disrupt each of these loci, deletion of the J region, deletion
35   of the J-C intron enhancer, and disruption of constant region
     coding sequences by the introduction of a stop codon. The last
     option is the most straightforward, in terms of DNA construct
     design. Elimination of the μ gene disrupts B-cell maturation
     thereby preventing class switching to any of the functional

heavy chain segments. The strategy for knocking out these loci
is outlined below.

To disrupt the mouse μ and κ genes, targeting vectors
are used based on the design employed by Jaenisch and co-

5   workers (Zijlstra, et al. (1989), Nature, 342, 435-438) for the
successful disruption of the mouse β2-microglobulin gene. The
neomycin resistance gene (neo), from the plasmid pMCIneo is
inserted into the coding region of the target gene. The
pMCIneo insert uses a hybrid viral promoter/enhancer sequence

10   to drive neo expression. This promoter is active in embryonic
stem cells. Therefore, neo can be used as a selectable marker
for integration of the knock-out construct. The HSV thymidine
kinase (tk) gene is added to the end of the construct as a
negative selection marker against random insertion events

15   (Zijlstra, et al., supra.).

The targeting vectors for disrupting the heavy chain
locus are illustrated in Fig. 7. The primary strategy for
disrupting the heavy chain locus is the elimination of the J
region. This region is fairly compact in the mouse, spanning

20   only 1.3 kb. To construct a gene targeting vector, a 15 kb
KpnI fragment containing all of the secreted A constant region
exons from mouse genomic library is isolated. The 1.3 kb J
region is replaced with the 1.1 kb insert from pMCIneo. The
HSV tk gene is then added to the 5' end of the KpnI fragment.

25   Correct integration of this construct, via homologous
recombination, will result in the replacement of the mouse J_H
region with the neo gene (Fig. 7). Recombinants are screened
by PCR, using a primer based on the neo gene and a primer
homologous to mouse sequences 5' of the KpnI site in the D

30   region.

Alternatively, the heavy-chain locus is knocked out by
disrupting the coding region of the μ gene. This approach
involves the same 15 kb KpnI fragment used in the previous
approach. The 1.1 kb insert from pMCIneo is inserted at a

35   unique BamHI site in exon II, and the HSV tk gene added to the
3' KpnI end. Double crossover events on either side of the neo
insert, that eliminate the tk gene, are then selected for.
These are detected from pools of selected clones by PCR

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amplification. One of the PCR primers is derived from neo
sequences and the other from mouse sequences outside of the
targeting vector. The functional disruption of the mouse
immunoglobulin loci is presented in the Examples.

5

Transgenic Non-Human Animals Containing Rearranged
Immunoglobulin Heavy and Light Transgenes

         A premise underlying the previously discussed
10   transgenic animals containing unrearranged mini-locus Ig
transgenes is that it is possible to generate a complete
antibody repertoire without including all of the variable gene
segments found in the natural immunoglobulin locus.
Theoretically, it is possible to reduce the number of different
15   sequences that contribute to the primary repertoire without
reducing the secondary repertoire. As long as there is enough
diversity in the primary repertoire to trigger a T-cell
dependent response for any given antigen, somatic hypermutation
should be capable of delivering a high affinity antibody
20   against that antigen.

         This concept is taken a step further in this aspect of
the invention wherein a full heterologous antibody repertoire
is generated entirely by somatic mutation. The antigen
combining site is created by the interface between the
25   amino-terminal heavy chain domain and the amino-terminal light
chain domain. The CDR1, 2 and 3 residues within each of these
domains that interact with the antigen are located on three
different loops that connect β strands. As previously
described, these regions have the greatest sequence diversity
30   between different antibody molecules recognizing different
antigens. Thus, the antibody repertoire is determined by
sequence diversity at CDR1, 2, and 3. The diversity at CDR1, 2,
and 3 that gives rise to a complete antibody repertoire comes
from three sources: recombinational diversity, junctional
35   diversity, and somatic mutation. Recombinational diversity at
CDR1 and 2 comes from the choice of different V segments
containing different CDR1 and 2 sequences. Recombinational
diversity at CDR 3 comes from the choice of different D and J
segments. Junctional diversity contributes only to CDR3

diversity, while somatic mutation, acting across the entire V region, contributes to diversity at all three complimentarity determining regions. Recombinational and junctional diversity together constitute the diversity of the primary repertoire

5 (Fig. 1). Thus VDJ joining generates a set of IgM expressing primary B-cells.

Any primary repertoire B-cell that expresses a cell surface IgM molecule with a certain minimal affinity for a foreign antigen, internalizes that antigen as IgM and cycle off

10 the cell surface. The antigen is then processed and associated peptides are presented on the cell surface by class II MHC molecules. If enough foreign antigen is presented at the cell surface this, triggers a T-cell response that in turn triggers the T-cell dependent maturation of the B-cell. This is the so-

15 called secondary response (Fig. 8). Part of this response involves the hypermutation of the variable portion of the immunoglobulin genes. Thus a B-cell clone undergoing a secondary response constantly gives rise to new clones with altered immunoglobulin molecules. Those clones with higher

20 affinities for the foreign antigen are selectively expanded by helper T-cells, giving rise to affinity maturation of the expressed antibody. Because somatic hypermutation takes place across the entire V region, there is no theoretical limit to the process of affinity maturation.

25 In this aspect of the invention, CDR1 and 2 diversity is not necessary for generating a complete antibody response. Rather, diversity at CDR3, created by VJ and VDJ joining provides sufficient minimal affinity to trigger the T-cell dependent maturation to give rise to high affinity antibodies

30 for a large number of different antigens. Thus, methods and transgenic animals are provided for generating a broad antibody repertoire without primary diversity. Such diversity relies on somatic mutation for the generation of antibody diversity. During the process of affinity maturation, somatic mutation

35 gives rise to a large number of clones with lower, rather than higher, affinities for the stimulating antigen. Most of these clones are not selected for and die off. However, if one of these clones has affinity for a new antigen that is also

present, this clone expands and undergoes affinity maturation
for the new antigen (Fig. 9). In this aspect of the invention,
a transgenic non-human animal, such as a mouse, with rearranged
human heavy and light chains combine to form an antibody that

5   has a low affinity for a known antigen. If this animal is
injected with the known antigen, its B-cells undergo a
secondary response leading to the production of high affinity
antibodies for that antigen. However, if this mouse is first
injected with a mixture of the known antigen and a new antigen,

10   and then subsequently challenged with the new antigen alone,
high affinity antibodies against the new antigen are produced
by the branching process described above. This approach has
two major advantages: first the transgene constructs are easy
to generate; and second, the rearranged transgenes are capable

15   of allelicly and isotypically excluding the rearrangement of
the endogenous mouse genes, thus making it unnecessary to
eliminate those genes by homologous recombination as previously
described.

       The first step in this embodiment of the invention is

20   the isolation of rearranged heavy and light chain genes from a
human hybridoma that expresses an IgM antibody directed against
a known antigen. The ideal hybridoma recognizes a readily
available antigen that is capable of generating a good mouse
T-cell response. There are a number of such human hybridomas

25   in existence, including several that react with promising
antigens such as tetanus toxoid, pseudomonas, or gram negative
bacteria (reviewed by James and Bourla (1987), J. Immunol.
Methods., 100, 5-40). The entire rearranged heavy chain gene
is isolated on a single piece of DNA (approximately 20 kb)

30   while the rearranged κ light chain gene, including the 3'
enhancer, is isolated on a second DNA fragment (about 20 kb).
Each of these fragments are pieced together from clones
isolated from a phage λ library made from DNA isolated from the
hybridoma. Two constructs are generated, a heavy chain

35   construct and a light chain construct.

       The heavy chain construct (Fig. 10) consists of the 20
kb hybridoma fragment, containing the rearranged IgM gene,
ligated to a 25 kb fragment that contains the human γ3 and γl

constant regions followed by a 700 bp fragment containing the rat heavy chain 3' enhancer (Pettersson, et al. (1990), Nature, 344, 165-168). The light chain construct consists of the intact 20 kb piece of DNA containing the rearranged κ chain and

5   3' enhancer. These two constructs are coinjected so that they are integrated at a single site in the mouse genome. Transgenic mice are tested by Northern blot analysis for expression of the transgene mRNA. FACS analysis is then carried-out on tail blood samples to detect cell surface

10   expression of the transgene encoded protein. Mice are then immunized with the antigen recognized by the original hybridoma. ELISA and FACS analysis are carried out on tail blood to detect class switching. Finally, the mice are tested for their ability to respond to a number of different antigens

15   by co-injecting a panel of antigens together with the original antigen. Tail blood are analyzed by ELISA to detect the production of high affinity human IgG antibodies directed against individual antigens.

To use this transgenic mouse to generate human

20   antibodies directed against a given antigen, that antigen preferably is first coinjected together with the antigen associated with the hybridoma from which the genes were isolated. This hybridoma associated antigen is referred to as the co-antigen (sometimes as a second antigen), and the new

25   antigen simply as the antigen (or first antigen). If possible, the second antigen is chemically cross-linked to the first antigen prior to injection. This causes the first antigen to be internalized and presented by the primary transgene presenting B-cells, thus ensuring the existence of a pool of

30   activated helper T-cells that recognize the first antigen. A typical immunization schedule is as follows. Day 1: Mice are injected ip with first antigen mixed with, or cross-linked to, second antigen in complete Freunds adjuvant. Day 14: first antigen (without second antigen) is injected ip in incomplete

35   Freunds adjuvant. Day 35: repeat injection with first antigen in incomplete Freunds. Day 45: Test for antibody response by ELISA on tail blood samples. Day 56: repeat injection of good

46

responders with antigen in incomplete Freunds. Day 59: Fuse spleens of good responders.

In an alternate aspect of this invention, the antigen recognized by the hybridoma from which the Ig genes were
5   isolated, is used as an immunogen. New transgenic hybridomas are then isolated from the immunized animal that express somatically mutated versions of the original antibody. These new antibodies will have a higher affinity for the original antigen. This antibody "sharpening" procedure can also be
10 applied to antibody genes generated by CDR grafting (E.P. Pub. No. 239400, published Sept. 30, 1987) or isolated from bacterial (W.D. Huse et al. (1989) Science, 246, 1275) or phage (T. Clackson et al. (1991) Nature, 352, 624) expression libraries.

15

Transgenic Non-Human Animals Containing
Rearranged and Unrearranged Immunoglobulin
Heavy and/or Light Transgene

20 The previous embodiments described the use of fully rearranged or fully unrearranged heavy and light immunoglobulin transgenes to produce transgenic non-human animals capable of producing a heterologous antibody. In a further aspect of the invention, transgenic animals contain at least one rearranged
25 and at least one unrearranged immunoglobulin transgene are produced by utilizing any of the aforementioned unrearranged and rearranged transgenes in combination to provide heavy and light transgenes in the transgenic animal. In this regard, the unrearranged transgene may comprise a heavy or light genomic or
30 mini-locus transgene construct with the rearranged transgene comprising an appropriate rearranged transgene. For example, if a unrearranged mini-locus light chain transgene is used, the appropriate other transgene is a fully rearranged heavy chain transgene. It is preferred, however, that the rearranged
35 transgene comprise a rearranged immunoglobulin light chain transgene and that the unrearranged transgene comprise an immunoglobulin heavy chain genomic or mini-locus transgene, most preferably an unrearranged heavy chain transgene with associated A and y constant regions.

47

The combination of rearranged and unrearranged
transgene provides an intermediate level of diversity within
the primary repertoire B-cells. Thus, although primary
diversity at CD1, CD2 and CD3 in the rearranged transgene is
5   fixed in the primary repertoire B-cell, the primary diversity
at the CDR1, CDR2 and CDR3 produced by the rearrangement of the
unrearranged transgene provides a population of primary
repertoire of B-cells having greater potential diversity than
the B-cell clone obtained when rearranged heavy and light
10   transgenes are used. Such primary diversity provides broadened
secondary diversity when such cells respond to foreign antigen
by way of somatic mutation.

Nucleic Acids

15   The nucleic acids, the term "substantial homology"
indicates that two nucleic acids, or designated sequences
thereof, when optimally aligned and compared, are identical,
with appropriate nucleotide insertions or deletions, in at
least about 80% of the nucleotides, usually at least about 90%
20   to 95%, and more preferably at least about 98 to 99.5% of the
nucleotides. Alternatively, substantial homology exists when
the segments will hybridize under selective hybridization
conditions, to the complement of the strand. The nucleic acids
may be present in whole cells, in a cell lysate, or in a
25   partially purified or substantially pure form. A nucleic acid
is "isolated" or "rendered substantially pure" when purified
away from other cellular components or other contaminants,
e.g., other cellular nucleic acids or proteins, by standard
techniques, including alkaline/SDS treatment, CsCl banding,
30   column chromatography, agarose gel electrophoresis and others
well known in the art. See, F. Ausubel, et al., ed. Current
Protocols in Molecular Biology, Greene Publishing and Wiley-
Interscience, New York (1987).
          The nucleic acid compositions of the present
35   invention, while often in a native sequence (except for
modified restriction sites and the like), from either cDNA,
genomic or mixtures may be mutated, thereof in accordance with
standard techniques to provide gene sequences. For coding

sequences, these mutations, may affect amino acid sequence as
desired. In particular, DNA sequences substantially homologous
to or derived from native V, D, J, constant, switches and other
such sequences described herein are contemplated (where
5   "derived" indicates that a sequence is identical or modified
from another sequence).

A nucleic acid is "operably linked" when it is placed
into a functional relationship with another nucleic acid
sequence. For instance, a promoter or enhancer is operably
10   linked to a coding sequence if it affects the transcription of
the sequence. With respect to transcription regulatory
sequences, operably linked means that the DNA sequences being
linked are contiguous and, where necessary to join two protein
coding regions, contiguous and in reading frame. For switch
15   sequences, operably linked indicates that the sequences are
capable of effecting switch recombination.

Specific Preferred Embodiments

A preferred embodiment of the invention is an animal
20   containing a single copy of the transgene described in Example
14 (pHC2) bred with an animal containing a single copy of the
transgene described in Example 16, and the offspring bred with
the JH deleted animal described in Examples 9 and 12. Animals
are bred to homozygosity for each of these three traits. Such
25   animals have the following genotype: a single copy (per
haploid set of chromosomes) of a human heavy chain unrearranged
mini-locus (described in Example 14), a single copy (per
haploid set of chromosomes) of a rearranged human κ light chain
construct (described in Example 16), and a deletion at each
30   endogenous mouse heavy chain locus that removes all of the
functional JH segments (described in Examples 9 and 12). Such
animals are bred with mice that are homozygous for the deletion
of the JH segments (Examples 9 and 12) to produce offspring
that are homozygous for the JH deletion and hemizygous for the
35   human heavy and light chain constructs. The resultant animals
are injected with antigens and used for production of human
monoclonal antibodies against these antigens.

49

B cells isolated from such an animal are monospecific with regards to the human heavy and light chains because they contain only a single copy of each gene. Furthermore, they will be monospecific with regards to human or mouse heavy

5   chains because both endogenous mouse heavy chain gene copies are nonfunctional by virtue of the deletion spanning the JH region introduced as described in Example 9 and 12. Furthermore, a substantial fraction of the B cells will be monospecific with regards to the human or mouse light chains

10 because expression of the single copy of the rearranged human κ light chain gene will allelically and isotypically exclude the rearrangement of the endogenous mouse κ and lambda chain genes in a significant fraction of B-cells.

The transgenic mouse of the preferred embodiment will

15 exhibit immunoglobulin production with a significant repertoire, ideally substantially similar to that of a native mouse. Thus, for example, when the endogenous Ig genes have been inactivated, the total immunoglobulin levels will range from about 0.1 to 10 mg/ml of serum, preferably 0.5 to 5 mg/ml,

20 ideally at least about 1.0 mg/ml. When a transgene capable of effecting a switch to IgG from IgM has been introduced into the transgenic mouse, the adult mouse ratio of serum IgG to IgM is preferably about 10:1. Of course, the IgG to IgM ratio will be much lower in the immature mouse. In general, greater than

25 about 10%, preferably 40 to 80% of the spleen and lymph node B cells express exclusively human IgG protein.

The repertoire will ideally approximate that shown in a non-transgenic mouse, usually at least about 10% as high, preferably 25 to 50% or more. Generally, at least about a

30 thousand different immunoglobulins (ideally IgG), preferably 10^4 to 10^6 or more, will be produced, depending primarily on the number of different V, J and D regions introduced into the mouse genome. These immunoglobulins will typically recognize about one-half or more of highly antigenic proteins, including,

35 but not limited to: pigeon cytochrome C, chicken lysozyme, pokeweed mitogen, bovine serum albumin, keyhole limpit hemocyanin, influenza hemagglutinin, staphylococcus protein A, sperm whale myoglobin, influenza neuraminidase, and lambda

50

repressor protein. Some of the immunoglobulins will exhibit an affinity for preselected antigens of at least about $10^{-7}\underline{M}^{-1}$, preferably $10^{-8}\underline{M}^{-1}$ to $10^{-9}\underline{M}^{-1}$ or greater.

 5   Although the foregoing describes a preferred embodiment of the transgenic animal of the invention, other embodiments are defined by the disclosure herein and more particularly by the transgenes described in the Examples. Four categories of transgenic animal may be defined:

10   I.   Transgenic animals containing an unrearranged heavy and rearranged light immunoglobulin transgene.
      II.   Transgenic animals containing an unrearranged heavy and unrearranged light immunoglobulin transgene
      III. Transgenic animal containing rearranged heavy and an
15   unrearranged light immunoglobulin transgene, and
      IV.   Transgenic animals containing rearranged heavy and rearranged light immunoglobulin transgenes.

       Of these categories of transgenic animal, the
20   preferred order of preference is as follows I > II > III > IV.
       Within each of these categories of the transgenic animal, a number of possible combinations are preferred. Such preferred embodiments comprise the following:

25   Category I
              (a)   Example 1 and 2 or 19 and 20 animal bred with Example 7 or 16 animal.
              (b)   Example 1 or 19 fragment coinjected with Example 7 or 16 fragment.
30   (c)   Example 5 (H, I or J) or 14, 17 or 21 animal bred with Example 7 or 16 animal.
              (d)   Example 5(H) or 14 construct coinjected with Example 7 or 16 construct.
              (e)   All of the above bred with the animal of Example
35   9 or 11, 12 or 13. Particularly preferred embodiments are all of the above bred the with animal of Example 9 or 12 or 13.

51

Category II

(a)   Example 1, 2, 19 or 20 animal bred with Example 6, 3, 4, 16, 22 or 23 animal.

(b)   Fragment in Example 1 or 19 coinjected with fragment in Example 2 or 20.

(c)   Example 5 (H, I or J) or 14, 17 or 21 animal bred with Example 6(B, C or D) or 16 animal.

(d)   Construct 5(H) or 14 coinjected with construct 6(B) or 16.

(e)   Animal of Example 1, 2, 19 or 20 bred with animal of Example 6(B, C or D) or 16.

(f)   Animal of Example 3, 4, 22 or 23 bred with animal of Example 5(H, I or J) or 14, 17 or 21.

(g)   All of the above bred with animal of Example 9, 10, 11, 12 or 13.

Category III

(a)   Example 3, 4, 22 or 23 animal bred with Example 8 or 15 animal.

(b)   Example 3 or 23 fragment coinjected with Example 8 or 15 fragment.

(c)   Example 6(B, C or D) or 16 animal bred with Example 8 or 15 animal.

(d)   Example 6(B) or 15 construct coinjected with Example 8 or 15 construct.

(e)   All of the above bred with animal of Example 9 to 13.

Category IV

(a)   Animal of Example 7 or 16, bred with animal of Example 8 or 15.

(b)   Construct of Example 7 or 16 coinjected with construct of Example 8 or 15.

(c)   All of the above bred with animal of Example 9 to 13.

The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

METHODS AND MATERIALS

Transgenic mice are derived according to Hogan, et al., "Manipulating the Mouse Embryo: A Laboratory Manual", Cold Spring Harbor Laboratory.

Embryonic stem cells are manipulated according to published procedures (Teratocarcinomas and embryonic stem cells: a practical approach, E.J. Robertson, ed., IRL Press, Washington, D.C., 1987; Zjilstra, et al. (1989), Nature, 342, 435-438; and Schwartzberg, P., et al. (1989), Science, 246, 799-803).

DNA cloning procedures are carried out according to J. Sambrook, et al. in Molecular Cloning: A Laboratory Manual, 2d ed., 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Oligonucleotides are synthesized on an Applied Bio Systems oligonucleotide synthesizer according to specifications provided by the manufacturer.

Hybridoma cells and antibodies are manipulated according to "Antibodies: A Laboratory Manual", Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988).

EXAMPLE 1

Genomic Heavy Chain Human Ig Transgene

This Example describes the cloning and microinjection of a human genomic heavy chain immunoglobulin transgene which is microinjected into a murine zygote.

Nuclei are isolated from fresh human placental tissue as described by Marzluff, W.F., et al. (1985), "Transcription and Translation: A Practical Approach", B.D. Hammes and S.J. Higgins, eds., pp. 89-129, IRL Press, Oxford). The isolated nuclei (or PBS washed human spermatocytes) are embedded in a low melting point agarose matrix and lysed with EDTA and proteinase κ to expose high molecular weight DNA, which is then digested in the agarose with the restriction enzyme NotI as described by M. Finney in Current Protocols in Molecular Biology (F. Ausubel, et al., eds. John Wiley & Sons, Supp. 4, 1988, Section 2.5.1).

The NotI digested DNA is then fractionated by pulsed
field gel electrophoresis as described by Anand, R., et al.
(1989) , <u>Nucl. Acids Res.</u>, <u>17</u>, 3425-3433. Fractions enriched
for the NotI fragment are assayed by Southern hybridization to
5   detect one or more of the sequences encoded by this fragment.
Such sequences include the heavy chain D segments, J segments,
μ and γ1 constant regions together with representatives of all
6 VH families (although this fragment is identified as 670 kb
fragment from HeLa cells by Berman, et al. (1988), <u>supra</u>., we
10   have found it to be as 830 kb fragment from human placental an
sperm DNA). Those fractions containing this NotI fragment (see
Fig. 4) are pooled and cloned into the NotI site of the vector
pYACNN in Yeast cells. Plasmid pYACNN is prepared by digestion
of pYAC-4 Neo (Cook, H., et al. (1988), <u>Nucleic Acids Res.</u>, <u>16</u>,
15   11817) with EcoRI and ligation in the presence of the
oligonucleotide 5' - AAT TGC GGC CGC - 3'.

YAC clones containing the heavy chain NotI fragment
are isolated as described by Brownstein, et al. (1989),
<u>Science</u>, <u>244</u>, 1348-1351, and Green, E., et al. (1990), <u>Proc.</u>
20   <u>Natl. Acad. Sci. USA</u>, <u>87</u>, 1213-1217. The cloned NotI insert is
isolated from high molecular weight yeast DNA by pulse field
gel electrophoresis as described by M. Finney, opcit. The DNA
is condensed by the addition of 1 mM spermine and microinjected
directly into the nucleus of single cell embryos previously
25   described.

EXAMPLE 2

Discontinuous Genomic Heavy Chain Ig Transgene

A 110 kb SpeI fragment of human genomic DNA containing
30   VH6, D segments, J segments, the μ constant region and part of
the γ constant region (see Fig. 4) is isolated by YAC cloning
as described in Example 1.

A 570 kb NotI fragment upstream of the 670-830 kb NotI
fragment described above containing multiple copies of VI
35   through V5 is isolated as described. (Berman, et al. (1988),
<u>supra</u> detected two 570 kb NotI fragments. Each of those
contain multiple V segments.)

54

The two fragments are coinjected into the nucleus of a
mouse single cell embryo as described in Example 1.

Coinjection of two different DNA fragments will
usually result in the integration of both fragments at the same
5   insertion site within the chromosome. Therefore, approximately
50% of the resulting transgenic animals that contain at least
one copy of each of the two fragments will have the V segment
fragment inserted upstream of the constant region containing
fragment. Of these animals, 50% will carry out V to DJ joining
10 by DNA inversion and 50% by deletion, depending on the
orientation of the 570 kb NotI fragment relative to the
position of the 110 kb SpeI fragment. DNA is isolated from
resultant transgenic animals and those animals found to be
containing both transgenes by Southern blot hybridization
15 (specifically, those animals containing both multiple human V
segments and human constant region genes) are tested for their
ability to express human immunoglobulin molecules.

EXAMPLE 3

20   Genomic κ Light Chain Human Ig Transgene
        Formed by In Vivo Homologous Recombination

A map of the human κ light chain has been described in
Lorenz, W., et al. (1987), Nucl. Acids Res., 15, 9667-9677 and
25 is depicted in Fig. 11.
A 450 kb XhoI to NotI fragment that includes all of
Ck, the 3' enhancer, all J segments, and at least five
different V segments (a) is isolated and microinjected into the
nucleus of single cell embryos as described in Example 1.

30

EXAMPLE 4

        Genomic κ Light Chain Human Ig Transgene
        Formed by In Vivo Homologous Recombination

35
A 750 kb MluI to NotI fragment that includes all of
the above plus at least 20 more V segments (b) is isolated as
described in Example 1 (see Fig. 11) and digested with BssHII
to produce a fragment of about 400 kb (c) .
40   The 450 kb XhoI to NotI fragment (a) plus the
approximately 400 kb MluI to BssHII fragment (c) have sequence

55

overlap defined by the BssHII and XhoI restriction sites shown
in Fig. 11. Homologous recombination of these two fragments
upon microinjection of a mouse zygote results in a transgene
containing at least an additional 15-20 V segments over that
5 found in the 450 kb XhoI/NotI fragment (Example 3).

EXAMPLE 5

Construction of Heavy Chain Mini-Locus

A.   Construction of pGP1 and pGP2

10   pBR322 is digested with EcoRI and StyI and ligated
with the following oligonucleotides to generate pGP1 which
contains a 147 base pair insert containing the restriction
sites shown in Fig. 13. The general overlapping of these
oligos is also shown in Fig. 13.

15   The oligonucleotides are:

oligo-1 5' - CTT GAG CCC GCC TAA TGA GCG GGC TTT
              TTT TTG CAT ACT GCG GCC - 3'

20 oligo-2 5' - GCA ATG GCC TGG ATC CAT GGC GCG CTA
                  GCA TCG ATA TCT AGA GCT CGA GCA -3'

oligo-3 5' - TGC AGA TCT GAA TTC CCG GGT ACC AAG
              CTT ACG CGT ACT AGT GCG GCC GCT -3'

25
oligo-4   5' - AAT TAG CGG CCG CAC TAG TAC GCG TAA
               GCT TGG TAC CCG GGA ATT - 3'

oligo-5   5' - CAG ATC TGC ATG CTC GAG CTC TAG ATA
30   TCG ATG CTA GCG CGC CAT GGA TCC - 3'

oligo-6   5' - AGG CCA TTG CGG CCG CAG TAT GCA AAA
               AAA AGC CGC TCA TTA GGC GGG CT - 3'

35
         This plasmid contains a large polylinker flanked by
rare cutting NotI sites for building large inserts that can be
isolated from vector sequences for microinjection. The plasmid
is based on pBR322 which is relatively low copy compared to the
40 pUC based plasmids (pGP1 retains the pBR322 copy number control
region near the origin of replication). Low copy number
reduces the potential toxicity of insert sequences. In
addition, pGP1 contains a strong transcription terminator
sequence derived from trpA (Christie, G.E., et al. (1981),
45 Proc. Natl. Acad. Sci. USA) inserted between the ampicillin

resistance gene and the polylinker. This further reduces the
toxicity associated with certain inserts by preventing
readthrough transcription coming from the ampicillin promoters.

Plasmid pGP2 is derived from pGP1 to introduce an

5   additional restriction site (SfiI) in the polylinker. pGP1 is
digested with MluI and SpeI to cut the recognition sequences in
the polylinker portion of the plasmid.

The following adapter oligonucleotides are ligated to
the thus digested pGP1 to form pGP2.

10

5' CGC GTG GCC GCA ATG GCC A 3'
5' CTA GTG GCC ATT GCG GCC A 3'

pGP2 is identical to pGP1 except that it contains an

15   additional Sfi I site located between the MluI and SpeI sites.
This allows inserts to be completely excised with SfiI as well
as with NotI.

B.   Construction of pRE3 (rat enhancer 3')

20   An enhancer sequence located downstream of the rat
constant region is included in the heavy chain constructs.

The heavy chain region 3' enhancer described by S.
Pettersson, et al. (1990), Nature, 344, 165-168) is isolated
and cloned. The rat IGH 3' enhancer sequence is PCR amplified

25   by using the following oligonucleotides:
5' CAG GAT CCA GAT ATC AGT ACC TGA AAC AGG GCT TGC 3'
5' GAG CAT GCA CAG GAC CTG GAG CAC ACA CAG CCT TCC 3'

The thus formed double stranded DNA encoding the 3'
enhancer is cut with BamHI and SphI and clone into BamHI/SphI

30   cut pGP2 to yield pRE3 (rat enhancer 3').

C.   Cloning of Human J-μ Region

A substantial portion of this region is cloned by
combining two or more fragments isolated from phage lambda

35   inserts. See Fig. 14.

A 6.3 kb BamHI/HindIII fragment that includes all
human J segments (Matsuda, et al. (1988), EMBO J., 7, 1047--
1051; Ravetech, et al. (1981), Cell, 27, 583-591) is isolated

57

from human genomic DNA library using the oligonucleotide GGA
CTG TGT CCC TGT GTG ATG CTT TTG ATG TCT GGG GCC AAG.

An adjacent 10 kb HindIII/BamII fragment that contains
enhancer, switch and constant region coding exons (Yasui, et

5   al. (1989), Eur. J. Immunol., 19, 1399-1403) is similarly
isolated using the oligonucleotide:
CAC CAA GTT GAC CTG CCT GGT CAC AGA CCT GAC CAC CTA TGA

An adjacent 3' 1.5 kb BamHI fragment is similarly
isolated using clone pMUM insert as probe (pMUM is 4 kb

10   EcoRI/HindIII fragment isolated from human genomic DNA library
with oligonucleotide:

CCT GTG GAC CAC CGC CTC CAC CTT CAT

CGT CCT CTT CCT CCT

mu membrane exon 1) and cloned into pUC19.

15   pGP1 is digested with BamHI and BglII followed by
treatment with calf intestinal alkaline phosphatase.

Fragments (a) and (b) from Fig. 14 are cloned in the
digested pGP1. A clone is then isolated which is oriented such
that 5' BamHI site is destroyed by BamHI/Bgl fusion. It is

20   identified as pMU (see Fig. 15). pMU is digested with BamHI
and fragment (c) from Fig. 14 is inserted. The orientation is
checked with HindIII digest. The resultant plasmid pHIG1 (Fig.
15) contains an 18 kb insert encoding J and Cμ segments.

25   D.   Cloning of Cμ Region

pGP1 is digested with BamHI and HindIII is followed by
treatment with calf intestinal alkaline phosphatase (Fig. 14).
The so treated fragment (b) of Fig. 14 and fragment (c) of Fig.
14 are cloned into the BamHI/HindIII cut pGP1. Proper

30   orientation of fragment (c) is checked by HindIII digestion to
form pCON1 containing a 12 kb insert encoding the Cμ region.

Whereas pHIG1 contains J segments, switch and μ
sequences in its 18 kb insert with an SfiI 3' site and a SpeI
5' site in a polylinker flanked by NotI sites, will be used for

35   rearranged VDJ segments. pCON1 is identical except that it
lacks the J region and contains only a 12 kb insert. The use
of pCON1 in the construction of fragment containing rearranged
VDJ segments will be described hereinafter.

58

E.   Cloning of γ-1 Constant Region (pREG2)

       The cloning of the human γ-1 region is depicted in

Fig. 16.

5   Yamamura, et al. (1986), Proc. Natl. Acad. Sci. USA,

83, 2152-2156 reported the expression of membrane bound human

γ-1 from a transgene construct that had been partially deleted

on integration.   Their results indicate that the 3' BamHI site

delineates a sequence that includes the transmembrane

10   rearranged and switched copy of the gamma gene with a V-C

intron of less than 5kb.   Therefore, in the unrearranged,

unswitched gene, the entire switch region is included in a

sequence beginning less than 5 kb from the 5' end of the first

γ-1 constant exon.   Therefore it is included in the 5' 5.3 kb

15   HindIII fragment (Ellison, J.W., et al. (1982), Nucleic Acids

Res., 10, 4071-4079).   Takahashi, et al. (1982), Cell, 29,

671-679 also reports that this fragment contains the switch

sequence, and this fragment together with the 7.7 kb HindIII to

BamHI fragment must include all of the sequences we need for

20   the transgene construct.

       Phage clones containing the γ-1 region are identified

and isolated using the following oligonucleotide which is

specific for the third exon of γ-I (CH3).

              5' TGA GCC ACG AAG ACC CTG AGG
                     TCA AGT TCA ACT GGT ACG TGG 3'
25   A 7.7 kb HindIII to BglII fragment (fragment (a) in

Fig. 16) is cloned into HindIII/BglII cut pRE3 to form pREG1.

The upstream 5.3 kb HindIII fragment (fragment (b) in Fig. 16)

is cloned into HindIII digested pREG1 to form pREG2.   Correct

30   orientation is confirmed by BamHI/SpeI digestion.

F.   Combining Cγ and Cμ

       The previously described plasmid pHIG1 contains human

J segments and the Cμ constant region exons.   To provide a

35   transgene containing the Cμ constant region gene segments,

pHIG1 was digested with SfiI (Fig. 15).   The plasmid pREG2 was

also digested with SfiI to produce a 13.5 kb insert containing

human Cγ exons and the rat 3' enhancer sequence.   These

sequences were combined to produce the plasmid pHIG3' (Fig. 17)

containing the human J segments, the human Cμ constant region,
the human Cγ1 constant region and the rat 3' enhancer contained
on a 31.5 kb insert.

     A second plasmid encoding human Cμ and human Cγ1

5   without J segments is constructed by digesting pCON1 with SfiI
and combining that with the SfiI fragment containing the human
Cγ region and the rat 3' enhancer by digesting pREG2 with SfiI.
The resultant plasmid, pCON (Fig. 17) contains a 26 kb
NotI/SpeI insert containing human Cμ, human γ1 and the rat 3'

10 enhancer sequence.

G.   <u>Cloning of D Segment</u>

     The strategy for cloning the human D segments is
depicted in Fig. 18. Phage clones from the human genomic

15 library containing D segments are identified and isolated using
probes specific for diversity region sequences (Y. Ichihara, et
al. (1988), <u>EMBO J</u>., <u>7</u>, 4141-4150). The following
oligonucleotides are used:

20 DXP1:   5' - TGG TAT TAC TAT GGT TCG GGG AGT TAT TAT
                 AAC CAC AGT GTC - 3'

    DXP4:   5' - GCC TGA AAT GGA GCC TCA GGG CAC AGT GGG
                  CAC GGA CAC TGT - 3'

25
    DN4:   5' - GCA GGG AGG ACA TGT TTA GGA TCT GAG GCC
                 GCA CCT GAC ACC - 3'

     A 5.2 kb XhoI fragment (fragment (b) in Fig. 18)
containing DLR1, DXP1, DXP'1, and DA1 is isolated from a phage

30 clone identified with oligo DXP1.

     A 3.2 kb XbaI fragment (fragment (c) in Fig. 18)
containing DXP4, DA4 and DK4 is isolated from a phage clone
identified with oligo DXP4.

     Fragments (b), (c) and (d) from Fig. 18 are combined

35 and cloned into the XbaI/XhoI site of pGP1 to form pHIG2 which
contains a 10.6 kb insert.

This cloning is performed sequentially. First, the

5.2 kb fragment (b) in Fig. 18 and the 2.2 kb fragment (d) of

Fig. 18 are treated with calf intestinal alkaline phosphatase

and cloned into pGP1 digested with XhoI and XbaI. The

5   resultant clones are screened with the 5.2 and 2.2 kb insert.

Half of those clones testing positive with the 5.2 and 2.2 kb

inserts have the 5.2 kb insert in the proper orientation as

determined by BamHI digestion. The 3.2 kb XbaI fragment from

Fig. 18 is then cloned into this intermediate plasmid

10   containing fragments (b) and (d) to form pHIG2 (Fig. 9). This

plasmid contains diversity segments cloned into the polylinker

with a unique 5' SfiI site and unique 3' SpeI site. The entire

polylinker is flanked by NotI sites.

15   H.   Construction of Heavy Chain Minilocus

          The following describes the construction of a human

heavy chain mini-locus which contain one or more V segments.

          An unrearranged V segment corresponding to that

identified as the V segment contained in the hybridoma

20   of Newkirk, et al. (1988), J. Clin. Invest., 81, 1511-1518, is

isolated using the following oligonucleotide:

                    5' - GAT CCT GGT TTA GTT AAA GAG GAT TTT
                         ATT CAC CCC TGT GTC - 3'

25   A restriction map of the unrearranged V segment is

determined to identify unique restriction sites which provide

upon digestion a DNA fragment having a length approximately 2

kb containing the unrearranged V segment together with 5' and

3' flanking sequences. The 5' prime sequences will include

30   promoter and other regulatory sequences whereas the 3' flanking

sequence provides recombination sequences necessary for V-DJ

joining. This approximately 3.0 kb V segment insert is cloned
into the polylinker of pGB2 to form pVH1.

pVH1 is digested with SfiI and the resultant fragment
is cloned into the SfiI site of pHIG2 to form a pHIG5'. Since
5   pHIG2 contains D segments only, the resultant pHIG5' plasmid
contains a single V segment together with D segments. The size
of the insert contained in pHIG5 is 10.6 kb plus the size of
the V segment insert.

The insert from pHIG5 is excised by digestion with
10 NotI and SpeI and isolated. pHIG3' which contains J, $C\mu$ and
$c\gamma$1 segments is digested with SpeI and NotI and the 3' kb
fragment containing such sequences and the rat 3' enhancer
sequence is isolated. These two fragments are combined and
ligated into NotI digested pGP1 to produce pHIG which contains
15 insert encoding a V segment, nine D segments, six functional J
segments, $C\mu$, $C\gamma$ and the rat 3' enhancer. The size of this
insert is approximately 43 kb plus the size of the V segment
insert.

20 I.   Construction of Heavy Chain Minilocus
          by Homologous Recombination

As indicated in the previous section, the insert of
pHIG is approximately 43 to 45 kb when a single V segment is
25 employed. This insert size is at or near the limit of that
which may be readily cloned into plasmid vectors. In order to
provide for the use of a greater number of V segments, the
following describes in vivo homologous recombination of
overlapping DNA fragments which upon homologous recombination
30 within a zygote or ES cell form a transgene containing the rat
3' enhancer sequence, the human $C\mu$, the human $C\gamma$1, human J
segments, human D segments and a multiplicity of human V
segments.

A 6.3 kb BamHI/HindIII fragment containing human J
35 segments (see fragment (a) in Fig. 14) is cloned into MluI/SpeI
digested pHIG5' using the following adapters:

                5' GAT CCA AGC AGT 3'

                5' CTA GAC TGC TTG 3'

40   5' CGC GTC GAA CTA 3'

5' AGC TTA GTT CGA 3'

The resultant is plasmid designated pHIG5'O (overlap).
5   The insert contained in this plasmid contains human V, D and J
segments. When the single V segment from pVH1 is used, the
size of this insert is approximately 17 kb plus 2 kb. This
insert is isolated and combined with the insert from pHIG3'
which contains the human J, Cμ, γ1 and rat 3' enhancer
10   sequences. Both inserts contain human J segments which provide
for approximately 6.3 kb of overlap between the two DNA
fragments. When coinjected into the mouse zygote, in vivo
homologous recombination occurs generating a transgene
equivalent to the insert contained in pHIG.

15   This approach provides for the addition of a
multiplicity of V segments into the transgene formed in vivo.
For example, instead of incorporating a single V segment into
pHIG5', a multiplicity of V segments contained on (1) isolated
genomic DNA, (2) ligated DNA derived from genomic DNA, or (3)
20   DNA encoding a synthetic V segment repertoire is cloned into
pHIG2 at the SfiI site to generate pHIG5' V_N. The J segments
fragment (a) of Fig. 14 is then cloned into pHIG5' V_N and the
insert isolated. This insert now contains a multiplicity of V
segments and J segments which overlap with the J segments
25   contained on the insert isolated from pHIG3'. When
cointroduced into the nucleus of a mouse zygote, homologous
recombination occurs to generate in vivo the transgene encoding
multiple V segments and multiple J segments, multiple D
segments, the Cμ region, the Cγ1 region (all from human) and
30   the rat 3' enhancer sequence.

J.   Construction of Heavy Chain Mini-Locus by
      Coinjection of Synthetic VH Region Fragment
      Together with Heavy Chain DJC Construct

35   Synthetic V_H region fragments are generated and
isolated as previously described. These fragments are
coinjected with the purified NotI insert of plasmid pHIG (or a
version of pHIG that does not contain any V segments). The
40   coinjected DNA fragments are inserted into a single site in the
chromosome. Some of the resulting transgenic animals will

63

contain transgene inserts that have synthetic V regions located
adjacent and upstream of the sequences in the pHIG construct.
These animals will have a larger human heavy chain primary
repertoire than the animals described in Example 5(H).

5

EXAMPLE 6

Construction of Light Chain Minilocus

10   A.   Construction of pEμl

        The construction of pEμl is depicted in Fig. 21. The
mouse heavy chain enhancer is isolated on the XbaI to EcoRI 678
bp fragment (J. Banerji, et al. (1983), Cell, 33, 729-740) from

15   phage clones using oligo:

        5' GAA TGG GAG TGA GGC TCT CTC ATA CCC
              TAT TCA GAA CTG ACT 3'

        This Eμ fragment is cloned into EcoRV/XbaI digested

20   pGP1 by blunt end filling in EcoRI site. The resultant plasmid
is designated pEmul.

B.   Construction Of κ Light chain Minilocus

        The κ construct contains at least one human $V_κ$

25   segment, all five human $J_κ$ segments, the human $J-C_κ$ enhancer,
human κ constant region exon, and, ideally, the human 3' κ
enhancer (K. Meyer, et al. (1989), EMBO J., 8, 1959-1964).
The κ enhancer in mouse is 9 kb downstream from $C_κ$. However,
it is as yet unidentified in the human. In addition, the

30   construct contains a copy of the mouse heavy chain J-Cμ
enhancers.
        The minilocus is constructed from four component
fragments:
        (a) A 16 kb SmaI fragment that contains the human $C_κ$

35   exon and the 3' human enhancer by analogy with the mouse locus
(fragment (a) in Fig. 20);
        (b) A 5' adjacent 5 kb SmaI fragment, which contains
all five J segments (fragment (b) in Fig. 20);
        (c) The mouse heavy chain intronic enhancer isolated

40   from pEμl (this sequence is included to induce expression of
the light chain construct as early as possible in B-cell

64

development. Because the heavy chain genes are transcribed
earlier than the light chain genes, this heavy chain enhancer
is presumably active at an earlier stage than the intronic κ
enhancer); and

5   (d) A fragment containing one or more V segments.
            The preparation of this construct is as follows.
Human placental DNA is digested with SmaI and fractionated on
agarose gel by electrophoresis. Similarly, human placental DNA
is digested with BamHI and fractionated by electrophoresis.
10 The 16 kb fraction is isolated from the SmaI digested gel and
the 11 kb region is similarly isolated from the gel containing
DNA digested with BamHI.
            The 16 kb SmaI fraction is cloned into Lambda FIX II
(Stratagene, La Jolla, California) which has been digested with
15 XhoI, treated with klenow fragment DNA polymerase to fill in
the XhoI restriction digest product. Ligation of the 16 kb
SmaI fraction destroys the SmaI sites and lases XhoI sites in
tact.
            The 11 kb BamHI fraction is cloned into λ EMBL3
20 (Strategene, La Jolla, California) which is digested with BamHI
prior to cloning.
            Clones from each library were probed with the Cκ
specific oligo:
                5' GAA CTG TGG CTG CAC CAT CTG TCT
25   TCA TCT TCC CGC CAT CTG 3'

            A 16 kb XhoI insert that was subcloned into the XhoI
cut pEμ1 so that Cκ is adjacent to the SmaI site. The
resultant plasmid was designated pKap1. See Fig. 22.
30   The above Cκ specific oligonucleotide is used to probe
the λ EMBL3/BamHI library to identify an 11 kb clone
corresponding to fragment (d) of Fig. 20. A 5 kb SmaI fragment
(fragment (b) in Fig. 20) is subcloned and subsequently
inserted into pKap1 digested with SmaI. Those plasmids
35 containing the correct orientation of J segments, Cκ and the Eμ
enhancer are designated pKap2.
            One or more Vκ segments are thereafter subcloned into
the MluI site of pKap2 to yield the plasmid pKapH which encodes
the human Vκ segments, the human Jκ segments, the human Cκ

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segments and the human Eμ enhancer. This insert is excised by
digesting pKapH with NotI and purified by agarose gel
electrophoresis. The thus purified insert is microinjected
into the pronucleus of a mouse zygote as previously described.

C.   Construction of κ Light Chain Minilocus by
     In Vivo Homologous Recombination

          The 11 kb BamHI fragment (fragment (d) in Fig. 20) is
cloned into BamHI digested pGP1 such that the 3' end is toward
the SfiI site. The resultant plasmid is designated pKAPint.
One or more Vκ segments is inserted into the polylinker between
the BamHI and SpeI sites in pKAPint to form pKapHV. The insert
of pKapHV is excised by digestion with NotI and purified. The
insert from pKap2 is excised by digestion with NotI and
purified. Each of these fragments contain regions of homology
in that the fragment from pKapHV contains a 5 kb sequence of
DNA that include the J_K segments which is substantially
homologous to the 5 kb SmaI fragment contained in the insert
obtained fron pKap2. As such, these inserts are capable of
homologously recombining when microinjected into a mouse zygote
to form a transgene encoding V_K, J_K and C_K.

D.   Construction of κ Light Chain Mini-Locus
     by Coinjection of Synthetic Vκ Region Fragment
     Together with Light Chain JC Construct

          Synthetic Vκ, region fragments are generated and
isolated as previously described. These DNA fragments are
coinjected with the purified NotI insert of plasmid pKap2 or
plasmid pKapH. The coinjected DNA fragments are inserted into
a single site in the chromosome. Some of the resulting
transgenics will contain transgene inserts that have synthetic
V regions located adjacent and upstream of the sequences in the
pKap2 or pKapH construct. These animals will have a larger
human κ light chain primary repertoire than those described in
Example 6(B).

66

EXAMPLE 7

Isolation of Genomic Clones
Corresponding to Rearranged and Expressed
Copies of Immunoglobulin κ Light Chain Genes

5

This example describes the cloning of immunoglobulin κ light chain genes from cultured cells that express an immunoglobulin of interest. Such cells may contain multiple

10   alleles of a given immunoglobulin gene. For example, a hybridoma might contain four copies of the κ light chain gene, two copies from the fusion partner cell line and two copies from the original B-cell expressing the immunoglobulin of interest. Of these four copies, only one encodes the

15   immunoglobulin of interest, despite the fact that several of them may be rearranged. The procedure described in this example allows for the selective cloning of the expressed copy of the κ light chain.

20   A.   Double Stranded cDNA

Cells from human hybridoma, or lymphoma, or other cell line that synthesizes either cell surface or secreted or both forms of IgM with a κ light chain are used for the isolation of polyA+ RNA. The RNA is then used for the synthesis of oligo dT

25   primed cDNA using the enzyme reverse transcriptase. The single stranded cDNA is then isolated and G residues are added to the 3' end using the enzyme polynucleotide terminal transferase. The Gtailed single-stranded cDNA is then purified and used as template for second strand synthesis (catalyzed by the enzyme

30   DNA polymerase) using the following oligonucleotide as a primer:

5' – GAG GTA CAC TGA CAT ACT GGC ATG CCC
CCC CCC CCC – 3'

35   The double stranded cDNA is isolated and used for determining the nucleotide sequence of the 5' end of the mRNAs encoding the heavy and light chains of the expressed immunoglobulin molecule. Genomic clones of these expressed genes are then isolated. The procedure for cloning the

40   expressed light chain gene is outlined in part B below.

B. <u>Light Chain</u>

The double stranded cDNA described in part A is denatured and used as a template for a third round of DNA synthesis using the following oligonucleotide primer:

5   5' - GTA CGC CAT ATC AGC TGG ATG AAG TCA TCA GAT
                GGC GGG AAG ATG AAG ACA GAT GGT GCA - 3'

This primer contains sequences specific for the constant portion of the κ light chain message (TCA TCA GAT GGC
10   GGG AAG ATG AAG ACA GAT GGT GCA) as well as unique sequences that can be used as a primer for the PCR amplification of the newly synthesized DNA strand (GTA CGC CAT ATC AGC TGG ATG AAG). The sequence is amplified by PCR using the following two oligonucleotide primers:

15
                5' - GAG GTA CAC TGA CAT ACT GGC ATG -3'
                5' - GTA CGC CAT ATC AGC TGG ATG AAG -3'

The PCR amplified sequence is then purified by gel
20   electrophoresis and used as template for dideoxy sequencing reactions using the following oligonucleotide as a primer:

                5' - GAG GTA CAC TGA CAT ACT GGC ATG -3'

The first 42 nucleotides of sequence will then be used to synthesize a unique probe for isolating the gene from which
25   immunoglobulin message was transcribed. This synthetic 42 nucleotide segment of DNA will be referred to below as o-kappa.

A Southern blot of DNA, isolated from the Ig expressing cell line and digested individually and in pairwise combinations with several different restriction endonucleases
30   including SmaI, is then probed with the 32-P labelled unique oligonucleotide o-kappa. A unique restriction endonuclease site is identified upstream of the rearranged V segment.

DNA from the Ig expressing cell line is then cut with SmaI and second enzyme (or BamHI or KpnI if there is SmaI site
35   inside V segment). Any resulting non-blunted ends are treated with the enzyme T4 DNA polymerase to give blunt ended DNA molecules. Then add restriction site encoding linkers (BamHI, EcoRI or XhoI depending on what site does not exist in fragment) and cut with the corresponding linker enzyme to give
40   DNA fragments with BamHI, EcoRI or XhoI ends. The DNA is then size fractionated by agarose gel electrophoresis, and the

fraction including the DNA fragment covering the expressed V
segment is cloned into lambda EMBL3 or Lambda FIX (Stratagene,
La Jolla, California). V segment containing clones are
isolated using the unique probe o-kappa. DNA is isolated from
5   positive clones and subcloned into the polylinker of pKap1.
The resulting clone is called pRKL.

EXAMPLE 8

Isolation of Genomic Clones
10   Corresponding to Rearranged Expressed Copies
of Immunoglobuling Heavy Chain μ Genes

This example describes the cloning of immunoglobulin
heavy chain μ genes from cultured cells of expressed and
15   immunoglobulin of interest. The procedure described in this
example allows for the selective cloning of the expressed copy
of a μ heavy chain gene.

Double-stranded cDNA is prepared and isolated as
described in part A of Example 7. The double-stranded cDNA is
20   denatured and used as a template for a third round of DNA
synthesis using the following oligonucleotide primer:

5' - GTA CGC CAT ATC AGC TGG ATG AAG ACA GGA GAC
GAG GGG GAA AAG GGT TGG GGC GGA TGC - 3'

25   This primer contains sequences specific for the
constant portion of the μ heavy chain message (ACA GGA GAC GAG
GGG GAA AAG GGT TGG GGC GGA TGC) as well as unique sequences
that can be used as a primer for the PCR amplification of the
newly synthesized DNA strand (GTA CGC CAT ATC AGC TGG ATG AAG).
30   The sequence is amplified by PCR using the following two
oligonucleotide primers: 5' - GAG GTA CAC TGA CAT ACT GGC ATG
- 3'

5' - GTA CTC CAT ATC AGC TGG ATG AAG - 3'

The PCR amplified sequence is then purified by gel
35   electrophoresis and used as template for dideoxy sequencing
reactions using the following oligonucleotide as a primer:

5' - GAG GTA CAC TGA CAT ACT GGC ATG - 3'

The first 42 nucleotides of sequence are then used to
synthesize a unique probe for isolating the gene from which
40   immunoglobulin message was transcribed. This synthetic 42
nucleotide segment of DNA will be referred to below as o-mu.

A Southern blot of DNA, isolated from the Ig expressing cell line and digested individually and in pairwise combinations with several different restriction endonucleases including MluI (MluI is a rare cutting enzyme that cleaves

5   between the J segment and mu CH1), is then probed with the 32-P labelled unique oligonucleotide o-mu. A unique restriction endonuclease site is identified upstream of the rearranged V segment.

DNA from the IG expressing cell line is then cut with

10 MluI and second enzyme. MluI or SpeI adapter linkers are then ligated onto the ends and cut to convert the upstream site to MluI or SpeI. The DNA is then size fractionated by agarose gel electrophoresis, and the fraction including the DNA fragment covering the expressed V segment is cloned directly into the

15 plasmid pGPI. V segment containing clones are isolated using the unique probe o-mu, and the insert is subcloned into MluI or MluI/SpeI cut plasmid pCON2. The resulting plasmid is called pRMGH.

20   EXAMPLE 9

Deletion of the Mouse Heavy Chain Gene
                 by Homologous Recombination

25   This example describes the deletion of the endogenous mouse heavy chain gene by homologous recombination in embryonic stem (ES) cells (Zjilstra, et al. (1989), Nature, 342, 435-438) followed by the transplantation of those ES cells into a mouse blastocyst embryo such that the ES cells colonize the germline

30 of the resultant chimeric mouse (Teratocarcinomas and embryonic stem cells: a practical approach, E.J. Robertson, ed., IRL press, Washington, D.C., 1987).

The construction of a DNA sequence that will homologously recombine into the mouse chromosome so as to

35 delete the heavy chain J segments, thus eliminating the possibility of successful gene rearrangement at the heavy chain locus. The design of this construct is outlined below.

Plasmid pGP1 is digested with the restriction endonucleases BamHI and BglII and religated to form the plasmid

70

pGP1dl. This plasmid is then used to build the so-called gene
knockout construct.

       To obtain sequences homologous to the desired target
region of the mouse genome, mouse genomic clones are isolated
5   from a phage library derived from non-lymphoid tissue (such as
liver) using the J_H specific oligonucleotide probe:

       5' - GGT CTA TGA TAG TGT GAC TAC TTT GAC TAC TGG
              GGC CAA GGC - 3'

10   A 3.5 kb KpnI to EcoRI fragment that hybridizes with
this probe is isolated from DNA derived from positive phage
clones. This fragment is subcloned into KpnI/EcoRI digested
pGP1dl to form the plasmid pMKO1.

       Neomycin resistance (Neo) and Herpes Simplex Virus
15 thymidine k inase (TK) genes for drug selection of recombinants
(M. Capecchi (1989), Science, 244, 1288-1292) are then isolated
as follows. The plasmid pGEM7(KJ1) (M.A. Rudnicki, 3/15/89) is
digested with HindIII and the ends blunted with the klenow form
of DNA pol I. The DNA is then cut with EcoRI and the pGKNeo
20 fragment is isolated and cloned into SphI/NaeI cut pMKO1 using
the following oligonucleotide as an adapter:

       5' - AATTCATG -3'

       The resulting plasmid is designated pMKO2. This
plasmid contains the neomycin resistance gene flanked by
25 sequences that flank the mouse J_H segments. This plasmid alone
can be used for deletion of the heavy chain gene.
Alternatively the Herpes TK gene can be added to the construct
to improve the frequency of homologous recombination events in
Neo resistant clones (M. Capecchi (1989), Science, 244,
30 1288-1292). This is done as follows. The EcoRI to HindIII
PGKTK fragment of pGEM7(TK) (M.A. Rudnicki) is isolated and
cloned into the KpnI site of pMKO2 using the following
oligonucleotide as adapters:

       5' - AATTGTAC - 3'

35   5' - AGCTGTAC - 3'

The resulting plasmid is designated pMKO3.

       To further improve the overall efficiency of
homologous recombination, a large segment of DNA that is
homologous to the target sequence is then added to the

construct. A 13 kb EcoRI fragment, that hybridizes with the Cμ specific oligonucleotide described below:

5' - GCA TCC TGG AAG GTT CAG ATG AAT ACC
TTG TAT GCA AAA TCC - 3'

5   This 12 kb fragment includes the Cμ coding exons, or a substantial portion of that fragment which includes the 5' EcoRI end, s isolated from a mouse genomic phage library and subcloned into the EcoRI site of pMKO3. The resultant plasmid is designated pMKO4.

10   The insert of pMKO4 is isolated by digestion with NotI and electroporated into ES cells. Homologous recombinant clones are isolated used to generate a J$_H$ deleted mouse as described by Zjilstra, et al. (1989), Nature, 342, 435-438.

15   EXAMPLE 10

Deletion of the Mouse Light Chain Gene
by Homologous Recombination

This example describes the deletion of the endogenous
20 mouse light chain gene by homologous recombination in embryonic stem cells (see previous Example).

A DNA sequence that homologously recombines into the mouse chromosome to delete the κ light chain constant region exon is constructed. The design of this construct is outlined
25 below.

A 2 kb BamHII to EcoRI thymidine kinase fragment from pGEM7(TK)Sal (M.A. Rudnicki, Whitehead Institute) is isolated and subcloned into the BamHI/SfiI digested pGP1 using the following oligonucleotide adapter:

30   5' - AATTTTG - 3'

The resulting plasmid is designated pKKO1.

To obtain sequences homologous to the desired target region of the mouse genome, mouse genomic clones are isolated from a phage library derived from non-lymphoid tissue (such as
35 liver) using the mouse κ light chain specific oligo designated o-MKC given below:

5' - GGC TGA TGC TGC ACC AAC TGT ATC CAT
CTT CCC ACC ATC CAG - 3'

DNA is isolated from positive clone and a 2.3 kb BglII
40 fragment (P.S. Neumaier and H.G. Zachau (1983), Nucl. Acids

Res., 11, 3631-3656) that hybridizes with probe o-MK3 is
isolated. The sequence of probe o-MK3 is as follows:

5' - CAT TCT GGG TAT GAA GAG CCC ACG TAT

CAA AGG TTA CAT TAG - 3'

5   This 2.3 kb BglII fragment is subcloned into BamHI
digested pKK01 such that the 3' end of the fragment is adjacent
to the polylinker SfiI site. The resulting plasmid is
designated pKK02.

        The 4 kb SphI to HpaI DNA fragment that hybridizes
10   with oligonucleotide o-MKC is isolated from positive phage
clone and subcloned into EcoRV to SphI digested plasmid pKK02.
The resulting plasmid is designated pKK03.

        A 2 kb SalI to EcoRI fragment of pGEM7(KJ1)Sal (M.A.
Rudnicki, 3/15/89) is isolated and cloned into the BssHII site
15   of plasmid pKK03 using linker adapters. This is carried out by
first ligating a mixture of the following three
oligonucleotides to the 2 kb SalI to EcoRI fragment:

5' - CAGCGCGC - 3'

5' - GATCGCGCGCTG - 3

5' - AATTGCGCGCTG - 3'

20   The ligation mixture is then digested with the enzyme
BssHII and ligated to BssHII digested plasmid pKK03. The
resulting plasmid is designated pKK04.

        The insert of pKK04 is isolated by digesting with NotI
25   and electroporated into ES cells. Homologous recombinant
clones are isolated and used to generate a C_κ deleted mouse as
described by Zjilstra, et al. (1989), Nature, 342, 435-438.

EXAMPLE 11

30   <u>Inactivation of the Mouse Kappa Light Chain Gene by Homologous
Recombination</u>

        This example describes the inactivation of the mouse
endogenous kappa locus by homologous recombination in embryonic
35   stem (ES) cells followed by introduction of the mutated gene
into the mouse germ line by injection of targeted ES cells
bearing an inactivated kappa allele into early mouse embryos
(blastocysts).

The strategy is to delete J_K and C_K by homologous recombination with a vector containing DNA sequences homologous to the mouse kappa locus in which a 4.5 kb segment of the locus, spanning the J_K gene and C_K segments, is deleted and

5   replaced by the selectable marker neo.

Construction of the kappa targeting vector

The plasmid pGEM7 (KJ1) (M. A. Rudnicki, Whitehead Institute) contains the neomycin resistance gene (neo), used

10   for drug selection of transfected ES cells, under the transcriptional control of the mouse phosphoglycerate kinase (pgk) promoter (XbaI/I/TaqI fragment; Adra, C.N. et al., (1987) Gene, 60, 65-74) in the cloning vector pGEM-72f(+). The plasmid also includes a heterologous polyadenylation site for

15   the neo gene, derived from the 3' region of the mouse pgk gene (PvuII/HindIII fragment; Boer, P.H., et al., (1990) Biochemical Genetics, 28, 299-308). This plasmid was used as the starting point for construction of the kappa targeting vector. The first step was to insert sequences homologous to the kappa

20   locus 3' of the neo expression cassette.

Mouse kappa chain sequences (Fig. 25a) were isolated from a genomic phage library derived from liver DNA using oligonucleotide probes specific for the C_K locus:

25   5'- GGC TGA TGC TGC ACC AAC TGT ATC CAT CTT CCC ACC ATC CAG -3'

and for the $J_K 5$ gene segment:

30   5'- CTC ACG TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA CGT AAG - 3'.

An 8 kb BglII/SacI fragment extending 3' of the mouse C_K segment was isolated from a positive phage clone in two

35   pieces, as a 1.2 kb BglII/SacI fragment and a 6.8 kb SacI fragment, and subcloned into BglII/SacI digested pGEM7 (KJ1) to generate the plasmid pNEO-K3' (Fig. 25b).

74

A 1.2 kb EcoRI/SphI fragment extending 5' of the J_κ region was also isolated from a positive phage clone. An SphI/XbaI/BglII/EcoRI adaptor was ligated to the SphI site of this fragment, and the resulting EcoRI fragment was ligated
5   into EcoRI digested pNEO-K3', in the same 5' to 3' orientation as the neo gene and the downstream 3' kappa sequences, to generate pNEO-K5'3' (Fig. 25c).

The Herpes Simplex Virus (HSV) thymidine kinase (TK) gene was then included in the construct in order to allow for
10 enrichment of ES clones bearing homologous recombinants, as described by Mansour et al. ((1988) Nature, 336, 348-352). The HSV TK cassette was obtained from the plasmid pGEM7 (TK) (M.A. Rudnicki), which contains the structural sequences for the HSV TK gene bracketed by the mouse pgk promoter and polyadenylation
15 sequences as described above for pGEM7 (KJ1). The EcoRI site of pGEM7 (TK) was modified to a BamHI site and the TK cassette was then excised as a BamHI/HindIII fragment and subcloned into pGP1b to generate pGP1b-TK. This plasmid was linearized at the XhoI site and the XhoI fragment from pNEO-K5'3', containing the
20 neo gene flanked by genomic sequences from 5' of J_κ and 3' of C_κ, was inserted into pGP1b-TK to generate the targeting vector J/C KI (Fig. 25d). The putative structure of the genomic kappa locus following homologous recombination with J/C K1 is shown in Fig. 25e.

25

Generation and analysis of ES cells with targeted inactivation of a kappa allele

AB-1 ES cells were grown on mitotically inactive SNL76/7 cell feeder layers (McMahon, A.P. and Bradley,A. (1990)
30 Cell, 62, 1073-1085) essentially as described (Robertson, E.J. (1987) in Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. E.J. Robertson, ed. (Oxford: IRL Press), p. 71-112).

The kappa chain inactivation vector J/C K1 was
35 digested with NotI and electroporated into AB-1 cells by the methods described (Hasty, P.R., et al. (1991) Nature, 350, 243-246). Electroporated cells were plated onto 100 mm dishes at a density of 2-5 x 10^6 cells/dish. After 24 hours, G418

(200μg/ml of active component) and FIAU (0.5μM) were added to
the medium, and drug-resistant clones were allowed to develop
over 10-11 days. Clones were picked, trypsinized, divided into
two portions, and further expanded. Half of the cells derived

5   from each clone were then frozen and the other half analyzed
for homologous recombination between vector and target
sequences.

          DNA analysis was carried out by Southern blot
hybridization. DNA was isolated from the clones as described

10   (Laird, P.W. et al., (1991) Nucl. Acids Res., 19,) digested
with XbaI and probed with the 800 bp EcoRI/XbaI fragment
indicated in Fig. 25e as the diagnostic probe. This probe
detects a 3.7 kb XbaI fragment in the wild type locus, and a
diagnostic 1.8 kb band in a locus which has homologously

15   recombined with the targeting vector (see Fig. 25a and e). Of
358 G418 and FIAU resistant clones screened by Southern blot
analysis, 4 displayed the 1.8 kb XbaI band indicative of a
homologous recombination at the kappa locus. These 4 clones
were further digested with the enzymes BglII, SacI, and PstI to

20   verify that the vector integrated homologously into one of the
kappa alleles. When probed with the diagnostic 800 bp
EcoRI/XbaI fragment, BglII, SacI, and PstI digests of wild type
DNA produce fragments of 4.1, 5.4, and 7 kb, respectively,
whereas the presence of a targeted kappa allele would be

25   indicated by fragments of 2.4, 7.5, and 5.7 kb, respectively
(see Fig. 25a and e). All 4 positive clones detected by the
XbaI digest showed the expected BglII, SacI, and PstI
restriction fragments diagnostic of a homologous recombination
at the kappa light chain.

30

     Generation of mice bearing the inactivated kappa chain
          The 4 targeted ES clones described in the previous
section were injected into C57Bl/6J blastocysts as described
(Bradley, A. (1987) in Teratocarcinomas and Embryonic Stem

35   Cells: A Practical Approach. E.J. Robertson, ed. (Oxford: IRL
Press), p. 113-151) and transferred into the uteri of
pseudopregnant females to generate chimeric mice representing a
mixture of cells derived from the input ES cells and the host

76

blastocyst.   Chimeric animals are visually identified by the
presence of agouti coat coloration, derived from the ES cell
line, on the black C57Bl/6J background.   The AB1 ES cells are
an XY cell line, thus male chimeras are bred with C57BL/6J

5   females and the offspring monitored for the presence of the
dominant agouti coat color.   Agouti offspring are indicative of
germline transmission of the ES genome.   The heterozygosity of
agouti offspring for the kappa chain inactivation is verified
by Southern blot analysis of DNA from tail biopsies using the

10   diagnostic probe utilized in identifying targeted ES clones.
Brother-sister matings of heterozygotes are then carried out to
generate mice homozygous for the kappa chain mutation.

EXAMPLE 12

15   Inactivation of the Mouse Heavy Chain Gene by Homologous
Recombination
This example describes the inactivation of the
endogenous murine immunoglobulin heavy chain locus by
homologous recombination in embryonic stem (ES) cells.   The

20   strategy is to delete the endogenous heavy chain J segments by
homologous recombination with a vector containing heavy chain
sequences from which the J_H region has been deleted and
replaced by the gene for the selectable marker neo.

25   Construction of a heavy chain targeting vector
Mouse heavy chain sequences containing the J_H region
(Fig. 26a) were isolated from a genomic phage library derived
from the D3 ES cell line (Gossler, et al., (1986) Proc. Natl.
Acad. Sci. U.S.A., 83, 9065-9069) using a J_H4 specific

30   oligonucleotide probe:
5'- ACT ATG CTA TGG ACT ACT GGG GTC AAG GAA CCT CAG TCA CCG -3'

A 3.5 kb genomic SacI/StuI fragment, spanning the J_H
region, was isolated from a positive phage clone and subcloned

35   into SacI/SmaI digested puc18.   The resulting plasmid was
designated puc18 J_H.   The neomycin resistance gene (neo), used
for drug selection of transfected ES cells, was derived from
the plasmid pGEM7 (KJ1).   The HindIII site in pGEM7 (KJ1) was
converted to a SalI site by addition of a synthetic adaptor,

77

and the neo expression cassette excised by digestion with
XbaI/SalI. The ends of the neo fragment were then blunted by
treatment with the Klenow form of DNA polI, and the neo
fragment was subcloned into the NaeI site of puc18 J_H,
5   generating the plasmid puc18 J_H-neo (Fig. 26b).

Further construction of the targeting vector was
carried out in a derivative of the plasmid pGP1b. pGP1b was
digested with the restriction enzyme NotI and ligated with the
following oligonucleotide as an adaptor:

10

5'- GGC CGC TCG ACG ATA GCC TCG AGG CTA TAA ATC TAG AAG AAT TCC
AGC AAA GCT TTG GC -3'

The resulting plasmid, called pGMT, was used to build
15 the mouse immunoglobulin heavy chain targeting construct.

The Herpes Simplex Virus (HSV) thymidine kinase (TK)
gene was included in the construct in order to allow for
enrichment of ES clones bearing homologous recombinants, as
described by Mansour et al. ((1988) Nature 336, 348-352). The
20 HSV TK gene was obtained from the plasmid pGEM7 (TK) by
digestion with EcoRI and HindIII. The TK DNA fragment was
subcloned between the EcoRI and HindIII sites of pGMT, creating
the plasmid pGMT-TK (Fig. 26c).

To provide an extensive region of homology to the
25 target sequence, a 5.9 kb genomic XbaI/XhoI fragment, situated
5' of the J_H region, was derived from a positive genomic phage
clone by limit digestion of the DNA with XhoI, and partial
digestion with XbaI. As noted in Fig. 26a and 26b, this XbaI
site is not present in genomic DNA, but is rather derived from
30 phage sequences immediately flanking the cloned genomic heavy
chain insert in the positive phage clone. The fragment was
subcloned into XbaI/XhoI digested pGMT-TK, to generate the
plasmid pGMT-TK-J_H5' (Fig. 26d).

The final step in the construction involved the
35 excision of the 3 kb EcoRI fragment from puc18 J_H-neo which
contained the neo gene and flanking genomic sequences. This
fragment was blunted by Klenow polymerase and subcloned into
the similarly blunted XhoI site of pGMT-TK-J_H5'. The resulting

78

construct, J_HKO1 (Fig. 26e), contains 6.9 kb of genomic
sequences flanking the J_H locus, with a 2.3 kb deletion
spanning the J_H region into which has been inserted the neo
gene. Fig. 25f shows the structure of an endogenous heavy
5   chain allele after homologous recombination with the targeting
construct.

EXAMPLE 13

Generation and analysis of targeted ES cells

10   AB-1 ES cells (McMahon, A.P. and Bradley,A. (1990)
Cell 62, 1073-1085) were grown on mitotically inactive SNL76/7
cell feeder layers essentially as described (Robertson, E.J.
(1987) Teratocarcinomas and Embryonic Stem Cells: A Practical
Approach. E.J. Robertson, ed. (Oxford: IRL Press), pp. 71-112).
15   The heavy chain inactivation vector J_HKO1 was digested
with NotI and electroporated into AB-1 cells by the methods
described (Hasty, P.R., et al. (1991) Nature 350, 243-246).
Electroporated cells were plated into 100 mm dishes at a
density of 2-5 x 10^6 cells/dish. After 24 hours, G418
20   (200mg/ml of active component) and FIAU (0.5mM) were added to
the medium, and drug-resistant clones were allowed to develop
over 8-10 days. Clones were picked, trypsinized, divided into
two portions, and further expanded. Half of the cells derived
from each clone were then frozen and the other half analyzed
25   for homologous recombination between vector and target
sequences.
               DNA analysis is carried out by Southern blot
hybridization. DNA is isolated from the clones as described
(Laird, P.W. et al., (1991) Nucl. Acids Res., 19.) digested
30   with HindIII and probed with the 500 bp EcoRI/StuI fragment
designated as the diagnostic probe in Fig. 26f. This probe
detects a HindIII fragment of 2.3 kb in the wild type locus,
whereas a 5.3 kb band is diagnostic of a targeted locus which
has homologously recombined with the targeting vector (see
35   Fig. 26a and f). Additional digests with the enzymes SpeI,
StuI, and BamHI are carried out to verify the targeted
disruption of the heavy chain allele.

EXAMPLE 14

Heavy Chain Minilocus Transgene

A.   Construction of plasmid vectors for cloning large DNA
sequences

5   1.   pGP1a

        The plasmid pBR322 was digested with EcoRI and StyI
and ligated with the following oligonucleotides:

oligo-42   5'- caa gag ccc gcc taa tga gcg ggc ttt ttt ttg cat

10   act gcg gcc gct -3'

oligo-43   5'- aat tag cgg ccg cag tat gca aaa aaa agc ccg ctc

           att agg cgg gct -3'

15   The resulting plasmid, pGP1a, is designed for cloning
very large DNA constructs that can be excised by the rare
cutting restriction enzyme NotI.   It contains a NotI
restriction site downstream (relative to the ampicillin
resistance gene, AmpR) of a strong transcription termination
20   signal derived from the trpA gene (Christie, G.E. et al. (1981)
Proc. Natl. Acad. Sci. USA, 78, 4180).   This termination signal
reduces the potential toxicity of coding sequences inserted
into the NotI site by eliminating readthrough transcription
from the AmpR gene.   In addition, this plasmid is low copy
25   relative to the pUC plasmids because it retains the pBR322 copy
number control region.   The low copy number further reduces the
potential toxicity of insert sequences and reduces the
selection against large inserts due to DNA replication.

30   2.   pGP1b

        pGP1a was digested with NotI and ligated with the
following oligonucleotides:

oligo-47   5'- ggc cgc aag ctt act gct gga tcc tta att aat cga

35   tag tga tct cga ggc -3'

oligo-48   5'- ggc cgc ctc gag atc act atc gat taa tta agg atc

           cag cag taa gct tgc -3'

80

        The resulting plasmid, pGP1b, contains a short
polylinker region flanked by NotI sites. This facilitates the
construction of large inserts that can be excised by NotI
digestion.

5

    3.   pGPe
The following oligonucleotides:

oligo-44 5'- ctc cag gat cca gat atc agt acc tga aac agg gct

10   tgc -3'

oligo-45 5'- ctc gag cat gca cag gac ctg gag cac aca cag cct
            tcc -3'

15   were used to amplify the immunoglobulin heavy chain 3' enhancer
(S. Petterson, et al. (1990) Nature, 344, 165-168) from rat
liver DNA by the polymerase chain reaction technique.
        The amplified product was digested with BamHI and SphI
and cloned into BamHI/SphI digested pNN03 (pNN03 is a pUC
20   derived plasmid that contains a polylinker with the following
restriction sites, listed in order: NotI, BamHI, NcoI, ClaI,
EcoRV, XbaI, SacI, XhoI, SphI, PstI, BglII, EcoRI, SmaI, KpnI,
HindIII, and NotI). The resulting plasmid, pRE3, was digested
with BamHI and HindIII, and the insert containing the rat Ig
25   heavy chain 3' enhancer cloned into BamHI/HindIII digested
pGP1b. The resulting plasmid, pGPe (Fig. 27 and Table 1),
contains several unique restriction sites into which sequences
can be cloned and subsequently excised together with the 3'
enhancer by NotI digestion.

81

AATTAGCggccgcctcgagatcactatcgattaattaaggatccagatatcagtacctgaaacagggcttgctcacaaca
tctctctctctgtctctctgtctctgtgtgtgtgtctctctctgtctctgtctctctctctgtctctctgtctctgtgtctc
tctctctctgtctctctctctgtctctctgtctctctgtctgtctctgtctctctgtctctgtctctctctctctctctctc
tctctctctctctctcacacacacacacacacacacacacacacacacccgccgagtgactcactctgtgcagggtggccc
tcggggcacatgcaaatggatgtttgttccatgcagaaaaacatgtttctcattctctgagccaaaaatagcatcaatga
ttcccccaccctgcagctgcaggttcaccccacctggccaggttgaccagctttggggatggggctgggggttccatgac
ccctaacggtgacattgaattcagtgtttttcccatttatcgacactgctggaatctgaccctaggagggaatgacaggag
ataggcaaggtccaaacaccccagggaagtgggagagacaggaaggctgtgtgtgctccaggtcctgtgcatgctgcaga
tctgaattcccgggtaccaagcttgcGGCCGCAGTATGCAAAAAAAAGCCCGCTCATTAGGCGGGCTCTTGGCAGAACAT
ATCCATCGCGTCCGCCATCTCCAGCAGCCGCACGCGGCGCATCTCGGGCAGCGTTGGGTCCTGGCCACGGGTGCGCATGA
TCGTGCTCCTGTCGTTGAGGACCCGGCTAGGCTGGCGGGGTTGCCTTACTGGTTAGCAGAATGAATCACCGATACGCGAG
CGAACGTGAAGCGACTGCTGCTGCAAAACGTCTGCGACCTGAGCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTA
AAGTCTGGAAACGCGGAAGTCAGCGCCCTGCACCATTATGTTCCGGATCTGCATCGCAGGATGCTGCTGGCTACCCTGTG
GAACACCTACATCTGTATTAACGAAGCGCTGGCATTGACCCTGAGTGATTTTTCTCTGGTCCCGCCGCATCCATACCGCC
AGTTGTTTACCCTCACAACGTTCCAGTAACCGGGCATGTTCATCATCAGTAACCCGTATCGTGAGCATCCTCTCTCGTTT
CATCGGTATCATTACCCCCATGAACAGAAATTCCCCCTTACACGGAGGCATCAAGTGACCAAACAGGAAAAAACCGCCCT
TAACATGGCCCGCTTTATCAGAAGCCAGACATTAACGCTTCTGGAGAAACTCAACGAGCTGGACGCGGATGAACAGGCAG
ACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACCGCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAAC
CTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGC
GTCAGCGGGTGTTGGCGGGTGTCGGGGCGCAGCCATGACCCAGTCACGTAGCGATAGCGGAGTGTATACTGGCTTAACTA
TGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACC
GCATCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCAC
TCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGC
CAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCT
CAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT
GTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTG
TAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCG
CCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGG
ATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGT
ATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCG
CTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTT
TCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCAC
CTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAAT
GCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATA
ACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTT
ATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTA
ATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTGCAGGCATCGTG
GTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTT
GTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTA
TGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCA
TTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAAC
TTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGA
TGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGG
CAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAG
CATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCA
CATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACG
AGGCCCTTTCGTCTTCAAG

Table 1   Sequence of vector pGPe.

82

B.   Construction of IgM expressing minilocus transgene, pIGM1

1.   Isolation of J-µ constant region clones and construction of
pJM1

A human placental genomic DNA library cloned into the
phage vector λEMBL3/SP6/T7 (Clonetech Laboratories, Inc., Palo
Alto, CA) was screened with the human heavy chain J region
specific oligonucleotide:

oligo-1   5'- gga ctg tgt ccc tgt gtg atg ctt ttg atg tct ggg
           gcc aag -3'

and the phage clone λ1.3 isolated. A 6 kb HindIII/KpnI
fragment from this clone, containing all six J segments as well
as D segment DHQ52 and the heavy chain J-µ intronic enhancer,
was isolated. The same library was screened with the human µ
specific oligonucleotide:

oligo-2   5'- cac caa gtt gac ctg cct ggt cac aga cct gac cac
           cta tga -3'

and the phage clone λ2.1 isolated. A 10.5 kb HindIII/XhoI
fragment, containing the µ switch region and all of the µ
constant region exons, was isolated from this clone. These two
fragments were ligated together with KpnI/XhoI digested pNN03
to obtain the plasmid pJM1.

2.   pJM2
A 4 kb XhoI fragment was isolated from phage clone
λ2.1 that contains sequences immediately downstream of the
sequences in pJM1, including the so called Σµ element involved
in µ deletion in certain IgD expressing B-cells (H. Yasui et
al. (1989) Eur. J. Immunol. 19, 1399). This fragment was
treated with the Klenow fragment of DNA polymerase I and
ligated to XhoI cut, Klenow treated, pJM1. The resulting
plasmid, pJM2 (Fig. 28), had lost the internal XhoI site but
retained the 3' XhoI site due to incomplete reaction by the
Klenow enzyme. pJM2 contains the entire human J region, the
heavy chain J-µ intronic enhancer, the µ switch region and all

of the μ constant region exons, as well as the two 0.4 kb
direct repeats, σμ and Σμ, involved in μ deletion.

3.   <u>Isolation of D region clones and construction of pDH1</u>

The following human D region specific oligonucleotide:

oligo-4   5'- tgg tat tac tat ggt tcg ggg agt tat tat aac cac
          agt gtc -3'

was used to screen the human placenta genomic library for D
region clones. Phage clones λ4.1 and λ4.3 were isolated. A
5.5 kb XhoI fragment, that includes the D elements D_{K1}, D_{N1}, and
D_{M2} (Y. Ichihara et al. (1988) <u>EMBO J.</u>, <u>7</u>, 4141), was isolated
from phage clone λ4.1. An adjacent upstream 5.2 kb XhoI
fragment, that includes the D elements D_{LR1}, D_{XP1}, $D_{XP\cdot1}$, and D_{A1},
was isolated from phage clone λ4.3. Each of these D region
XhoI fragments were cloned into the SalI site of the plasmid
vector pSP72 (Promega, Madison, WI) so as to destroy the XhoI
site linking the two sequences. The upstream fragment was then
excised with XhoI and SmaI, and the downstream fragment with
EcoRV and XhoI. The resulting isolated fragments were ligated
together with SalI digested pSP72 to give the plasmid pDH1.
pDH1 contains a 10.6 kb insert that includes at least 7 D
segments and can be excised with XhoI (5') and EcoRV (3').

4.   <u>pCOR1</u>
The plasmid pJM2 was digested with Asp718 (an
isoschizomer of KpnI) and the overhang filled in with the
Klenow fragment of DNA polymerase I. The resulting DNA was
then digested with ClaI and the insert isolated. This insert
was ligated to the XhoI/EcoRV insert of pDH1 and XhoI/ClaI
digested pGPe to generate pCOR1 (Fig. 29).

5.   <u>pVH251</u>
A 10.3 kb genomic HindIII fragment containing the two
human heavy chain variable region segments V_H251 and V_H105 (C.G.

Humphries et al. (1988) <u>Nature</u> <u>331</u>, 446) was subcloned into
pSP72 to give the plasmid pVH251.

6.   <u>pIGM1</u>

5   The plasmid pCOR1 was partially digested with XhoI and
the isolated XhoI/SalI insert of pVH251 cloned into the
upstream XhoI site to generate the plasmid pIGM1 (Fig. 30).
pIGM1 contains 2 functional human variable region segments, at
least 8 human D segments all 6 human J_H segments, the human $J-\mu$
10   enhancer, the human $\sigma\mu$ element, the human μ switch region, all
of the human μ coding exons, and the human $\Sigma\mu$ element, together
with the rat heavy chain 3' enhancer, such that all of these
sequence elements can be isolated on a single fragment, away
from vector sequences, by digestion with NotI and microinjected
15   into mouse embryo pronuclei to generate transgenic animals.

C.   <u>Construction of IqM and IqG expressing minilocus transgene,</u>
<u>pHC1</u>
1.   <u>Isolation of γ constant region clones</u>
20   The following oligonucleotide, specific for human Ig g
constant region genes:

oligo-29 5'- cag cag gtg cac acc caa tgc cca tga gcc cag aca
             ctg gac -3'

25

was used to screen the human genomic library. Phage clones
l29.4 and λ29.5 were isolated. A 4 kb HindIII fragment of
phage clone λ29.4, containing a γ switch region, was used to
probe a human placenta genomic DNA library cloned into the
30   phage vector lambda FIX™ II (Stratagene, La Jolla, CA). Phage
clone λSg1.13 was isolated. To determine the subclass of the
different γ clones, dideoxy sequencing reactions were carried
out using subclones of each of the three phage clones as
templates and the following oligonucleotide as a primer:

35

oligo-67 5'- tga gcc cag aca ctg gac -3'
            Phage clones λ29.5 and λSγ1.13 were both determined to
be of the γ1 subclass.

2. __pγe1__

A 7.8 kb HindIII fragment of phage clone λ29.5, containing the γ1 coding region was cloned into pUC18. The

5   resulting plasmid, pLT1, was digested with XhoI, Klenow treated, and religated to destroy the internal XhoI site. The resulting clone, pLT1xk, was digested with HindIII and the insert isolated and cloned into pSP72 to generate the plasmid clone pLT1xks. Digestion of pLT1xks at a polylinker XhoI site

10   and a human sequence derived BamHI site generates a 7.6 kb fragment containing the γ1 constant region coding exons. This 7.6 kb XhoI/BamHI fragment was cloned together with an adjacent downstream 4.5 kb BamHI fragment from phage clone λ29.5 into XhoI/BamHI digested pGPe to generate the plasmid clone pγe1.

15   pγe1 contains all of the γ1 constant region coding exons, together with 5 kb of downstream sequences, linked to the rat heavy chain 3' enhancer.

3. __pγe2__

20   A 5.3 kb HindIII fragment containing the γ1 switch region and the first exon of the pre-switch sterile transcript (P. Sideras et al. (1989) _International Immunol. 1_, 631) was isolated from phage clone λSγ1.13 and cloned into pSP72 with the polylinker XhoI site adjacent to the 5' end of the insert,

25   to generate the plasmid clone pSγ1s. The XhoI/SalI insert of pSγ1s was cloned into XhoI digested pγe1 to generate the plasmid clone pγe2 (Fig. 31). pγe2 contains all of the γ1 constant region coding exons, and the upstream switch region and sterile transcript exons, together with 5 kb of downstream

30   sequences, linked to the rat heavy chain 3' enhancer. This clone contains a unique XhoI site at the 5' end of the insert. The entire insert, together with the XhoI site and the 3' rat enhancer can be excised from vector sequences by digestion with NotI.

35

4. __pHC1__

The plasmid pIGM1 was digested with XhoI and the 43 kb insert isolated and cloned into XhoI digested pge2 to generate

the plasmid pHC1 (Fig. 30). pHC1 contains 2 functional human
variable region segments, at least 8 human D segments all 6
human J_H segments, the human J-μ enhancer, the human $\sigma\mu$
element, the human μ switch region, all of the human μ coding

5   exons, the human $\Sigma\mu$ element, and the human $\gamma1$ constant region,
including the associated switch region and sterile transcript
associated exons, together with the rat heavy chain 3'
enhancer, such that all of these sequence elements can be
isolated on a single fragment, away from vector sequences, by

10   digestion with NotI and microinjected into mouse embryo
pronuclei to generate transgenic animals.

D.   Construction of IgM and IgG expressing minilocus transgene,
pHC2

15   1.   Isolation of human heavy chain V region gene VH49.8
         The human placental genomic DNA library lambda, FIX™
II, Stratagene, La Jolla, CA) was screened with the following
human VH1 family specific oligonucleotide:

20   oligo-49 5'- gtt aaa gag gat ttt att cac ccc tgt gtc ctc tcc
         aca ggt gtc -3'

         Phage clone λ49.8 was isolated and a 6.1 kb XbaI
fragment containing the variable segment VH49.8 subcloned into

25   pNNO3 (such that the polylinker ClaI site is downstream of
VH49.8 and the polylinker XhoI site is upstream) to generate
the plasmid pVH49.8. An 800 bp region of this insert was
sequenced, and VH49.8 found to have an open reading frame and
intact splicing and recombination signals, thus indicating that

30   the gene is functional (Table 2).

```
TTCCTCAGGC AGGATTTAGG GCTTGGTCTC TCAGCATCCC ACACTTGTAC      50

AGCTGATGTG GCATCTGTGT TTTCTTTCTC ATCCTAGATC AAGCTTTGAG     100

CTGTGAAATA CCCTGCCTCA TGAATATGCA AATAATCTGA GGTCTTCTGA     150

GATAAATATA GATATATTGG TGCCCTGAGA GCATCACATA ACAACCAGAT     200

TCCTCCTCTA AAGAAGCCCC TGGAGCACA GCTCATCACC ATGGACTGGA     250
                                            MetAspTrpT
CCTGGAGGTT CCTCTTTGTG GTGGCAGCAG CTACAGgtaa ggggcttcct    300
hrTrpArgPh eLeuPheVal ValAlaAlaA laThr
agtcctaagg ctgaggaagg gatcctggtt tagttaaaga ggattttatt    350

caccctgtg tcctctccac agGTGTCCAG TCCCAGGTCC AGCTGGTGCA      400
                        GlyValGln SerGlnValG lnLeuValGl
GTCTGGGGCT GAGGTGAAGA AGCCTGGGTC CTCGGTGAAG GTCTCCTGCA     450
nSerGlyAla GluValLysL ysProGlySe rSerValLys ValSerCysL
AGGCTTCTGG AGGCACCTTC AGCAGCTATG CTATCAGCTG GGTGCGACAG     500
ysAlaSerGl yGlyThrPhe SerSerTyrA laIleSerTr pValArgGln
GCCCCTGGAC AAGGGCTTGA GTGGATGGGA AGGATCATCC CTATCCTTGG     550
AlaProGlyG lnGlyLeuGl uTrpMetGly ArgIleIleP roIleLeuGl
TATAGCAAAC TACGCACAGA AGTTCCAGGG CAGAGTCACG ATTACCGCGG     600
yIleAlaAsn TyrAlaGlnL ysPheGlnGl yArgValThr IleThrAlaA
ACAAATCCAC GAGCACAGCC TACATGGAGC TGAGCAGCCT GAGATCTGAG     650
spLysSerTh rSerThrAla TyrMetGluL euSerSerLe uArgSerGlu
GACACGGCCG TGTATTACTG TGCGAGAGAT ACAGTGTGA AACCCACATC      700
AspThrAlaV alTyrTyrCy sAlaArg
CTGAGAGTCT CAGAAACTCT GAGGGAGAAG GCAGCTGTGC CGGGCTGAGG     750

AGATGACAGG GTTTATTAGG TTTAAGGCTG TTTACAAAAT GGGTTATATA     800

TTTGAGAAAA AA                                             812
```

Table 2   Sequence of human V_H I family gene V_H 49.8

2. pV2

A 4 kb XbaI genomic fragment containing the human V$_H$IV
family gene V$_H$4-21 (I. Sanz et al. (1989) EMBO J., 8, 3741),
subcloned into the plasmid pUC12, was excised with SmaI and
5 HindIII, and treated with the Klenow fragment of polymerase I.
The blunt ended fragment was then cloned into ClaI digested,
Klenow treated, pVH49.8. The resulting plasmid, pV2, contains
the human heavy chain gene VH49.8 linked upstream of VH4-21 in
the same orientation, with a unique SalI site at the 3' end of
10 the insert and a unique XhoI site at the 5' end.

3. pSγ1-5'

A 0.7 kb XbaI/HindIII fragment (representing sequences
immediately upstream of, and adjacent to, the 5.3 kb γ1 switch
15 region containing fragment in the plasmid pγe2) together with
the neighboring upstream 3.1 kb XbaI fragment were isolated
from the phage clone λSg1.13 and cloned into HindIII/XbaI
digested pUC18 vector. The resulting plasmid, pSγ1-5',
contains a 3.8 kb insert representing sequences upstream of the
20 initiation site of the sterile transcript found in B-cells
prior to switching to the γ1 isotype (P. Sideras et al. (1989)
International Immunol., 1, 631). Because the transcript is
implicated in the initiation of isotype switching, and upstream
cis-acting sequences are often important for transcription
25 regulation, these sequences are included in transgene
constructs to promote correct expression of the sterile
transcript and the associated switch recombination.

4. pVGE1

30 The pSγ1-5' insert was excised with SmaI and HindIII,
treated with Klenow enzyme, and ligated with the following
oligonucleotide linker:

5'- ccg gtc gac cgg -3'

35

The ligation product was digested with SalI and ligated to SalI
digested pV2. The resulting plasmid, pVP, contains 3.8 kb of
γ1 switch 5' flanking sequences linked downstream of the two

human variable gene segments VH49.8 and VH4-21 (see Table 2).
The pVP insert is isolated by partial digestion with SalI and
complete digestion with XhoI, followed by purification of the
15 kb fragment on an agarose gel. The insert is then cloned
5   into the XhoI site of pγe2 to generate the plasmid clone pVGE1
(Fig. 32). pVGE1 contains two human heavy chain variable gene
segments upstream of the human γ1 constant gene and associated
switch region. A unique SalI site between the variable and
constant regions can be used to clone in D, J, and μ gene
10   segments. The rat heavy chain 3' enhancer is linked to the 3'
end of the γ1 gene and the entire insert is flanked by NotI
sites.

5. pHC2

15   The plasmid clone pVGE1 is digested with SalI and the
XhoI insert of pIGM1 is cloned into it. The resulting clone,
pHC2 (Fig. 30), contains 4 functional human variable region
segments, at least 8 human D segments all 6 human J_H segments,
the human J-m enhancer, the human σμ element, the human μ
20   switch region, all of the human μ coding exons, the human Σμ
element, and the human γ1 constant region, including the
associated switch region and sterile transcript associated
exons, together with 4 kb flanking sequences upstream of the
sterile transcript initiation site. These human sequences are
25   linked to the rat heavy chain 3' enhancer, such that all of the
sequence elements can be isolated on a single fragment, away
from vector sequences, by digestion with NotI and microinjected
into mouse embryo pronuclei to generate transgenic animals. A
unique XhoI site at the 5' end of the insert can be used to
30   clone in additional human variable gene segments to further
expand the recombinational diversity of this heavy chain
minilocus.

E. Transgenic mice

35   The NotI inserts of plasmids pIGM1 and pHC1 were
isolated from vector sequences by agarose gel electrophoresis.
The purified inserts were microinjected into the pronuclei of
fertilized (C57BL/6 x CBA)F2 mouse embryos and transferred the

surviving embryos into pseudopregnant females as described by
Hogan et al. (B. Hogan, F. Costantini, and E. Lacy, Methods of
Manipulating the Mouse Embryo, 1986, Cold Spring Harbor
Laboratory, New York). Mice that developed from injected

5   embryos were analyzed for the presence of transgene sequences
by Southern blot analysis of tail DNA. Transgene copy number
was estimated by band intensity relative to control standards
containing known quantities of cloned DNA. At 3 to 8 weeks of
age, serum was isolated from these animals and assayed for the

10 presence of transgene encoded human IgM and IgG1 by ELISA as
described by Harlow and Lane (E. Harlow and D. Lane.
Antibodies: A Laboratory Manual, 1988, Cold Spring Harbor
Laboratory, New York). Microtiter plate wells were coated with
mouse monoclonal antibodies specific for human IgM (clone AF6,

15 #0285, AMAC, Inc. Westbrook, ME) and human IgG1 (clone JL512,
#0280, AMAC, Inc. Westbrook, ME). Serum samples were serially
diluted into the wells and the presence of specific
immunoglobulins detected with affinity isolated alkaline
phosphatase conjugated goat anti-human Ig (polyvalent) that had

20 been pre-adsorbed to minimize cross-reactivity with mouse
immunoglobulins. Fig. 33 shows the results of an ELISA assay
for the presence of human IgM and IgG1 in the serum of two
animals that developed from embryos injected with the transgene
insert of plasmid pHC1. One of the animals (#18) was negative

25 for the transgene by Southern blot analysis, and showed no
detectable levels of human IgM or IgG1. The second animal
(#38) contained approximately 5 copies of the transgene, as
assayed by Southern blotting, and showed detectable levels of
both human IgM and IgG1. The results of ELISA assays for 11

30 animals that developed from transgene injected embryos is
summarized in the table below (Table 3).

Table 3.   Detection of human IgM and IgG1 in the serum of transgenic animals by ELISA assay

animal #	injected transgene	approximate transgene copy # (per cell)	human IgM	human IgG1
6	pIGM1	1	+ +	-
7	pIGM1	0	-	-
9	pIGM1	0	-	-
10	pIGM1	0	-	-
12	pIGM1	0	-	-
15	pIGM1	10	+ +	-
18	pHC1	0	-	-
19	pHC1	1	-	-
21	pHC1	<1	-	-
26	pHC1	2	+ +	+
38	pHC1	5	+ +	+

Table 3 shows a correlation between the presence of
integrated transgene DNA and the presence of transgene encoded
immunoglobulins in the serum. Two of the animals that were
found to contain the pHC1 transgene did not express detectable

5   levels of human immunoglobulins. These were both low copy
animals and may not have contained complete copies of the
transgenes, or the animals may have been genetic mosaics
(indicated by the <1 copy per cell estimated for animal #21),
and the transgene containing cells may not have populated the

10   hematopoetic lineage. Alternatively, the transgenes may have
integrated into genomic locations that are not conducive to
their expression. The detection of human IgM in the serum of
pIGM1 transgenics, and human IgM and IgG1 in pHC1 transgenics,
indicates that the transgene sequences function correctly in

15   directing VDJ joining, transcription, and isotype switching.

EXAMPLE 15

Rearranged Heavy Chain Transgenes

A.   Isolation of Rearranged Human Heavy Chain VDJ segments.

20   Two human leukocyte genomic DNA libraries cloned into
the phage vector 1EMBL3/SP6/T7 (Clonetech Laboratories, Inc.,
Palo Alto, CA) are screened with a 1 kb PacI/HindIII fragment
of λ1.3 containing the human heavy chain J-μ intronic enhancer.
Positive clones are tested for hybridization with a mixture of

25   the following V_H specific oligonucleotides:

oligo-7   5'-tca gtg aag gtt tcc tgc aag gca tct gga tac acc ttc
          acc-3'

30   oligo-8   5'-tcc ctg aga ctc tcc tgt gca gcc tct gga ttc acc ttc
          agt-3'

Clones that hybridized with both V and J-μ probes are
isolated and the DNA sequence of the rearranged VDJ segment

35   determined.

B. <u>Construction of rearranged human heavy chain transgenes</u>

Fragments containing functional VJ segments (open
reading frame and splice signals) are subcloned into the
plasmid vector pSP72 such that the plasmid derived XhoI site is
5   adjacent to the 5' end of the insert sequence. A subclone
containing a functional VDJ segment is digested with XhoI and
PacI (PacI, a rare-cutting enzyme, recognizes a site near the
J-m intronic enhancer), and the insert cloned into XhoI/PacI
digested pHC2 to generate a transgene construct with a
10   functional VDJ segment, the J-μ intronic enhancer, the μ switch
element, the μ constant region coding exons, and the γ1
constant region, including the sterile transcript associated
sequences, the γ1 switch, and the coding exons. This transgene
construct is excised with NotI and microinjected into the
15   pronuclei of mouse embryos to generate transgenic animals as
described above.

<u>EXAMPLE 16</u>

<u>Light Chain Transgenes</u>
20   A.   <u>Construction of Plasmid vectors</u>
1.   Plasmid vector pGP1c
Plasmid vector pGP1a is digested with NotI and the
following oligonucleotides ligated in: .

25   oligo-81 5'-ggc cgc atc ccg ggt ctc gag gtc gac aag ctt tcg agg
atc cgc-3'

oligo-82 5'-ggc cgc gga tcc tcg aaa gct tgt cga cct cga gac ccg
30   gga tgc-3'

The resulting plasmid, pGP1c, contains a polylinker with XmaI,
XhoI, SalI, HindIII, and BamHI restriction sites flanked by
NotI sites.

35

2.   Plasmid vector pGP1d
Plasmid vector pGP1a is digested with NotI and the
following oligonucleotides ligated in:

oligo-87 5'-ggc cgc tgt cga caa gct tat cga tgg atc ctc gag tgc
          -3'

oligo-88 5'-ggc cgc act cga gga tcc atc gat aag ctt gtc gac agc
          -3'

The resulting plasmid, pGP1d, contains a polylinker with SalI,
HindIII, ClaI, BamHI, and XhoI restriction sites flanked by
NotI sites.

B.   <u>Isolation of Jκ and Cκ clones</u>
        A human placental genomic DNA library cloned into the
phage vector λEMBL3/SP6/T7 (Clonetech Laboratories, Inc., Palo
Alto, CA) was screened with the human kappa light chain J
region specific oligonucleotide:

oligo-36 5'- cac ctt cgg cca agg gac acg act gga gat taa acg
          taa gca -3'

and the phage clones 136.2 and 136.5 isolated. A 7.4 kb XhoI
fragment that includes the Jκ1 segment was isolated from 136.2
and subcloned into the plasmid pNNO3 to generate the plasmid
clone p36.2. A neighboring 13 kb XhoI fragment that includes
Jk segments 2 through 5 together with the Cκ gene segment was
isolated from phage clone 136.5 and subcloned into the plasmid
pNNO3 to generate the plasmid clone p36.5. Together these two
clones span the region beginning 7.2 kb upstream of Jκ1 and
ending 9 kb downstream of Cκ.

C.   <u>Construction of rearranged light chain transgenes</u>
1.   pCK1, a Cκ vector for expressing rearranged variable
segments
        The 13 kb XhoI insert of plasmid clone p36.5
containing the Cκ gene, together with 9 kb of downstream
sequences, is cloned into the SalI site of plasmid vector pGP1c
with the 5' end of the insert adjacent to the plasmid XhoI
site. The resulting clone, pCK1 can accept cloned fragments
containing rearranged VJκ segments into the unique 5' XhoI

site. The transgene can then be excised with NotI and purified from vector sequences by gel electrophoresis. The resulting transgene construct will contain the human J-Cκ intronic enhancer and may contain the human 3' κ enhancer.

5

2. pCK2, a Cκ vector with heavy chain enhancers for expressing rearranged variable segments

A 0.9 kb XbaI fragment of mouse genomic DNA containing the mouse heavy chain J-μ intronic enhancer (J. Banerji et al. (1983) Cell 33, 729-740) was subcloned into pUC18 to generate the plasmid pJH22.1. This plasmid was linearized with SphI and the ends filled in with klenow enzyme. The klenow treated DNA was then digested with HindIII and a 1.4 kb MluI(klenow)/HindIII fragment of phage clone λ1.3 (previous example), containing the human heavy chain J-μ intronic enhancer (A. Hayday et al. (1984) Nature 307, 334-340), to it. The resulting plasmid, pMHE1, consists of the mouse and human heavy chain J-μ intronic enhancers ligated together into pUC18 such that they are excised on a single BamHI/HindIII fragment. This 2.3 kb fragment is isolated and cloned into pGP1c to generate pMHE2. pMHE2 is digested with SalI and the 13 kb XhoI insert of p36.5 cloned in. The resulting plasmid, pCK2, is identical to pCK1, except that the mouse and human heavy chain J-μ intronic enhancers are fused to the 3' end of the transgene insert. To modulate expression of the final transgene, analogous constructs can be generated with different enhancers, i.e. the mouse or rat 3' kappa or heavy chain enhancer (K. Meyer and M.S. Neuberger, (1989) EMBO J., 8, 1959-1964; S. Petterson, et al. (1990) Nature, 344, 165-168).

2. Isolation of rearranged kappa light chain variable segments

Two human leukocyte genomic DNA libraries cloned into the phage vector λEMBL3/SP6/T7 (Clonetech Laboratories, Inc., Palo Alto, CA) were screened with the human kappa light chain J region containing 3.5 kb XhoI/SmaI fragment of p36.5. Positive clones were tested for hybridization with the following Vκ specific oligonucleotide:

96

oligo-65 5'-agg ttc agt ggc agt ggg tct ggg aca gac ttc act ctc
         acc atc agc-3'

     Clones that hybridized with both V and J probes are isolated
5   and the DNA sequence of the rearranged VJκ segment determined.

     3.   Generation of transgenic mice containing rearranged human
     light chain constructs.
            Fragments containing functional VJ segments (open
10   reading frame and splice signals) are subcloned into the unique
     XhoI sites of vectors pCK1 and pCK2 to generate rearranged
     kappa light chain transgenes. The transgene constructs are
     isolated from vector sequences by digestion with NotI. Agarose
     gel purified insert is microinjected into mouse embryo
15   pronuclei to generate transgenic animals. Animals expressing
     human kappa chain are bred with heavy chain minilocus
     containing transgenic animals (EXAMPLE 14) to generate mice
     expressing fully human antibodies.
            Because not all VJκ combinations may be capable of
20   forming stable heavy-light chain complexes with a broad
     spectrum of different heavy chain VDJ combinations, several
     different light chain transgene constructs are generated, each
     using a different rearranged VJk clone, and transgenic mice
     that result from these constructs are bred with heavy chain
25   minilocus transgene expressing mice. Peripheral blood, spleen,
     and lymph node lymphocytes are isolated from double transgenic
     (both heavy and light chain constructs) animals, stained with
     fluorescent antibodies specific for human and mouse heavy and
     light chain immunoglobulins (Pharmingen, San Diego, CA) and
30   analyzed by flow cytometry using a FACScan analyzer (Becton
     Dickinson, San Jose, CA). Rearranged light chain transgenes
     constructs that result in the highest level of human
     heavy/light chain complexes on the surface of the highest
     number of B cells, and do not adversely affect the immune cell
35   compartment (as assayed by flow cytometric analysis with B and
     T cell subset specific antibodies), are selected for the
     generation of human monoclonal antibodies.

97

D.   Construction of unrearranged light chain minilocus

transgenes

1.   pJCK1, a Jκ, Cκ containing vector for constructing

minilocus transgenes

5   The 13 kb Cκ containing XhoI insert of p36.5 is

treated with klenow enzyme and cloned into HindIII digested,

klenow treated, plasmid pGP1d. A plasmid clone is selected

such that the 5' end of the insert is adjacent to the vector

derived ClaI site. The resulting plasmid, p36.5-1d, is

10   digested with ClaI and klenow treated. The Jκ1 containing 7.4

kb XhoI insert of p36.2 is then klenow treated and cloned into

the ClaI, klenow treated p36.5-1d. A clone is selected in

which the p36.2 insert is in the same orientation as the p36.5

insert. This clone, pJCK1 (Fig. 34), contains the entire human

15   Jκ region and Cκ, together with 7.2 kb of upstream sequences

and 9 kb of downstream sequences. The insert also contains the

human J-Cκ intronic enhancer and may contain a human 3' κ

enhancer. The insert is flanked by a unique 3' SalI site for

the purpose of cloning additional 3' flanking sequences such as

20   heavy chain or light chain enhancers. A unique XhoI site is

located at the 5' end of the insert for the purpose of cloning

in unrearranged Vκ gene segments. The unique SalI and XhoI

sites are in turn flanked by NotI sites that are used to

isolate the completed transgene construct away from vector

25   sequences.

2.   Isolation of unrearranged Vκ gene segments and generation

of transgenic animals expressing human Ig light chain protein

          The Vκ specific oligonucleotide, oligo-65 (discussed

30   above), is used to probe a human placental genomic DNA library

cloned into the phage vector 1EMBL3/SP6/T7 (Clonetech

Laboratories, Inc., Palo Alto, CA). Variable gene segments

from the resulting clones are sequenced, and clones that appear

functional are selected. Criteria for judging functionality

35   include: open reading frames, intact splice acceptor and donor

sequences, and intact recombination sequence. DNA fragments

containing selected variable gene segments are cloned into the

unique XhoI site of plasmid pJCK1 to generate minilocus

constructs. The resulting clones are digested with NotI and
the inserts isolated and injected into mouse embryo pronuclei
to generate transgenic animals. The transgenes of these
animals will undergo V to J joining in developing B-cells.

5   Animals expressing human kappa chain are bred with heavy chain
minilocus containing transgenic animals (EXAMPLE 14) to
generate mice expressing fully human antibodies.

EXAMPLE 17

10 Synthetic Heavy Chain Variable Region
            This example is outlined in Fig. 35.

A. Construction of Cloning Vector pVHf

1.   pGP1f

15   The plasmid pGP1a (previous example) is digested with
NotI and the following oligonucleotides are ligated to it:

oligo-"a" 5'-ggc cgc atg cta ctc gag tgc aag ctt ggc cat cca-3'
oligo-"b" 5'-ggc ctg gat ggc caa gct tgc act cga gta gca tgc-3'

20

The resulting plasmid, pGP1f, contains SphI, XhoI, and HindIII
sites flanked by NotI and SfiI sites.

2. pVHf

25   The human V_H-V family variable gene segment V_H251
(C.G. Humphries et al. (1988) Nature, 331, 446) together with
approximately 2.4 kb of 5' flanking sequences and approximately
1.4 kb of 3' flanking sequences was isolated on a 4.2 kb
SphI/HindIII fragment from the plasmid clone pVH251 (previous

30 example) and cloned into the plasmid vector pSelect™-1 (Promega
Corp., Madison, WI). The 5' flanking sequences, together with
the promoter, first exon and first intron of V_H251, are
amplified by polymerase chain reaction (PCR) from this template
using the following oligonucleotides:

35

oligo-83 5'-cag ctc gag ctc ggc aca ggc gcc tgt ggg-3'
oligo-84 5'-ctc tag agt cga cct gca ggc-3'

The 3' flanking sequences are amplified by PCR using
the following oligonucleotides:

oligo-85 5'-agc ctc gag ccc gtc taa aac cct cca cac-3'

oligo-86 5'-ggt gac act ata gaa tac tca agc-3'

The amplified 5' sequences are digested with SphI and XhoI, and
the 3' sequences digested with HindIII and XhoI. The resulting
fragments are cloned together into the plasmid pGP1f to
generate plasmid pVHf. Plasmid pVHf contains the cis acting
regulatory elements that control transcription of $V_H 251$,
together with the signal sequence encoding first exon. pVHf is
used as an expression cassette for heavy chain variable
sequences. Such sequences are cloned into the KasI/XhoI
digested plasmid as described below.

B.   Isolation of Variable Gene Coding Sequences
1.   Amplification of expressed V_H gene cDNA sequences
            Poly (A)⁺ RNA is isolated from human peripheral blood
lymphocytes (PBL). First strand cDNA is synthesized with
reverse transcriptase, using oligo-(dT) as a primer. The first
strand cDNA is isolated and tailed with oligo (dG) using
terminal transferase. The 5' sequences of IgM transcripts are
then specifically amplified by a modification of the method of
Frohman et al. (1988, Proc. Natl. Acad. Sci. USA, 85, 8998).
Oligo-(dC)$_{13}$ and the following oligonucleotide:

oligo-69 5'-gga att ctc aca gga gac gag-3'

are used as 5' and 3' primers, respectively, in a polymerase
chain reaction with dG-tailed first strand PBL cDNA. Oligo-69
is complimentary to sequences encoding amino acids 11-17 of the
IgM constant domain. Therefore these primers will amplify DNA
fragments of approximately 0.6 kb that include expressed V_H
gene sequences.

2.   Back-conversion of cDNA sequences into germline form
          The following oligonucleotide:
oligo-"c" 5'-ctg acg act ctg tat ggc gcc (ct)a(cg) t(cg)(ct)
          (cg)ag (ag)t(cg) ca(ag) ct(gt) gtg (cg)a(ag) tc(gt)
5   gg(gt)-3'

is annealed to denatured, PCR amplified, IgM 5' sequences.
Oligo-"c" includes a 21 nucleotide nondegenerate sequence that
includes a KasI site, followed by a 30 nucleotide degenerate
10 sequence that is homologous to the 5' end of the second exon of
many human V_H segments (Genbank; Los Alamos, NM). The primer
is extended with DNA polymerase and the product isolated from
unused primer by size fractionation. The product is then
denatured and annealed to the following oligonucleotide:

15

oligo-"d" 5'-ggg ctc gag gct ggt ttc tct cac tgt gtg t(cgt)t
          (acgt)(ag)(ct) aca gta ata ca(ct) (ag)g(ct)-3'

Oligo-"d" includes a 30 nucleotide nondegenerate sequence that
20 includes an XhoI site and part of the V to DJ recombination
sequence, followed by a 21 nucleotide degenerate sequence that
is complimentary to the the sequence encoding the last seven
amino acids in framework region three of many human variable
gene segments. The annealed oligonucleotide is then extended
25 with DNA polymerase and the product isolated from unused primer
by size fractionation. Single rounds of DNA synthesis followed
by removal of primers are carried out to ensure the sequence
integrity of individual variable gene fragments. The product
of oligo-"d" primer extension is amplified by PCR using the
30 following two oligonucleotides as primers:

oligo-"e" 5'-ctg acg act ctg tat ggc gcc-3'

oligo-"f" 5'-ggg ctc gag gct ggt ttc tct-3'

35

The resulting 0.36 kb PCR product is purified by gel
electrophoresis and digested with the restriction enzymes KasI
and XhoI. Digestion products are then cloned into KasI/XhoI

digested pVHf to generate a library of expressed variable gene
sequences in germline configuration. Ligation into the KasI
site of pVHf recreates the splice acceptor site at the 5' end
of the second exon, while ligation into the XhoI site recreates
5   the recombination signal at the 3' end of the variable gene
segment. Alternative versions of degenerate oligonucleotides
"c" and "d" are used to amplify different populations of
variable genes, and generate germline-configuration libraries
representing those different populations (Genbank; Los Alamos,
10   NN).

C.   Construction of Synthetic Locus
           The entire library of synthetic germline-configuration
V_H genes is grown up together and plasmid DNA isolated. The
15   medium copy plasmid pVHf, which includes a strong transcription
terminator between the ampicillin resistance gene and the
cloning site, is designed to minimize the expansion of
particular clones within the library. Plasmid DNA is digested
with SfiI, treated with calf intestinal phosphatase to remove
20   5' phosphate groups, and then digested with NotI. The calf
intestinal phosphatase is removed prior to NotI digestion so
that only the SfiI ends are dephosphorylated. The digested DNA
is then isolated from vector sequences by agarose gel
electrophoresis and ligated to the following oligonucleotides:

25

oligo-"g" 5'-ggc cta act gag cgt ccc ata ttg aga acc tcc -3'

oligo-"h" 5'-ggt tct caa tat ggg acg ctc agt ta-3'

30   Oligo-"h" is kinased while oligo-"g" is left unphosphorylated.
The ligation reaction is carried out with a large molar excess
of oligonucleotides so that all of the V gene fragment NotI
ends will be ligated to oligonucleotides and not other V region
fragments. Because the SfiI ends are not self compatible, the
35   V segments will concatenate in the same orientation such that
each V segment is separated by a single oligonucleotide spacer
unit from the next V segment.

Large concatomers are sized by electrophoresis and
isolated from agarose gels. The size fractionated concatomers
are then directly coinjected into mouse embryo pronuclei
together with D-J-C containing DNA fragments (such as the pHCl
5   or pHC2 inserts) to generate transgenic animals with large
primary repertoires. Alternatively, the concatomers are cloned
into a plasmid vector such as pGPf.

                          EXAMPLE 18

10 Generation of Lymphoid Cell Receptor Subset Specific
Antibodies.

          The inoculation of mice with xenogeneic (i.e. human)
immunoglobulins (B-cell receptors) or T-cell receptors leads
predominantly to the generation of mouse antibodies directed
15 against particular epitopes (dominant epitopes) that shared by
all or most immunoglobulins or T-cell receptors of a given
species, but differ between species. It is therefore difficult
to isolate antibodies that distinguish particular subsets of B
or T cell receptors (e.g., idiotypes or variable region
20 families). However, the transgenic mouse expressing human
immunoglobulins (described in the above examples) will be
immunologically tolerant of those shared B-cell epitopes and
will therefore be useful for generating antibodies that
distinguish subsets of human immunoglobulins. This concept is
25 extended by generating transgenic mice expressing human T-cell
receptor coding sequences and breeding these mice with the
human immunoglobulin transgenic mice. Such mice are inoculated
with isolates containing human T-cell receptor proteins and
monoclonal antibodies are generated that recognize T-cell
30 receptor subsets.

          Studies have demonstrated that there is a limited
variability of T cell antigen receptors involved in certain
autoimmune diseases (T.F. Davies et al. (1991) New England J.
Med., 325, 238). Because of this limited variability, it is
35 possible to generate human monoclonal antibodies that
specifically recognize that subset of human T cells which is
auto-reactive.

A. Generation of B-cell subset specific antibodies

      Human immunoglobulin expressing transgenic mice are inoculated with immunoglobulins isolated from a healthy donor or from a patient with a B-cell malignancy expressing a high

5   level of a single immunoglobulin type (Miller et al. (1982) New Eng. J. Med. 306, 517-522). Monoclonal antibody secreting hybridomas are generated as described by Harlow and Lane (E. Harlow and D. Lane. Antibodies: A Laboratory Manual. 1988. Cold Spring Harbor Laboratory, New York). Individual hybridomas

10 that secrete human antibodies that specifically recognize B-cell subsets are selected.

B. Transgenic mice expressing human T-cell receptor sequences.

      DNA fragments containing intact and fully rearranged

15 human T-cell receptor (TCR) α and β genes are coinjected into mouse embryo pronuclei to generate transgenic animals. Transgenic animals are assayed by FACS analysis for the expression of both transgenes on the surface of their T-cells. Animals are selected that express only low levels of the human

20 α and β TCR chains on a fraction of their T-cells. Only low level expression is required to obtain immunological tolerance, and high level expression will disturb the animal's immune system and interfere with the ability to mount an immune response required for the generation monoclonal antibodies.

25 Alternatively, because correct tissue or cell type specific expression is not required to obtain immunologic tolerance, TCR α and β chain cDNA clones are inserted into transgene expression cassettes (T. Choi et al. (1991) Mol. Cell. Biol., 11, 3070-3074) under the control of non-TCR transcription

30 signals. TCR α and β chain cDNA transgene constructs are coinjected into mouse embryo pronuclei to generate transgenic animals. Ectopic expression of the TCR chains will not result in cell surface expression because the TCR is a multichain complex (H. Clevers et al. 1988 Ann. Rev. Immunol., 6,

35 629-662); however, cell surface expression is not required for antigen presentation (Townsend et al. (1986) Nature, 324, 575-577) and tolerance induction.

104

T-cell receptor α and β chain transgenic mice are bred
with human immunoglobulin expressing transgenic mice to
generate mice that are useful for generating human monoclonal
antibodies that recognize specific subsets of human T-cells.
5   Such mice are inoculated with T-cell derived proteins isolated
from a healthy donor or from a patient with a T-cell malignancy
expressing a single TCR type. Monoclonal antibody secreting
hybridomas are generated and individual hybridomas that secrete
human antibodies that specifically recognize B-cell subsets are
10   selected.

                          EXAMPLE 19
Genomic Heavy Chain Human Ig Transgene
            This Example describes the cloning of a human genomic
15   heavy chain immunoglobulin transgene which is then introduced
into the murine germline via microinjection into zygotes or
integration in ES cells.
            Nuclei are isolated from fresh human placental tissue
asdescribed by Marzluff, W.F., et al. (1985), Transcription and
20   Translation: A Practical Approach, B.D. Hammes and S.J.
Higgins, eds., pp. 89-129, IRL Press, Oxford). The isolated
nuclei (or PBS washed human spermatocytes) are embedded in 0.5%
low melting point agarose blocks and lysed with 1 mg/ml
proteinase K in 500mM EDTA, 1% SDS for nuclei, or with 1mg/ml
25   proteinase K in 500mM EDTA, 1% SDS, 10mM DTT for spermatocytes
at 50°C for 18 hours. The proteinase K is inactivated by
incubating the blocks in 40μg/ml PMSF in TE for 30 minutes at
50°C, and then washing extensively with TE. The DNA is then
digested in the agarose with the restriction enzyme NotI as
30   described by M. Finney in Current Protocols in Molecular
Biology (F. Ausubel et al., eds. John Wiley & Sons, Supp. 4,
1988, e.g., Section 2.5.1).
            The NotI digested DNA is then fractionated by pulsed
field gel electrophoresis as described by Anand, R. et al.
35   (1989), Nuc. Acids Res., 17, 3425-3433. Fractions enriched for
the NotI fragment are assayed by Southern hybridization to
detect one or more of the sequences encoded by this fragment.
Such sequences include the heavy chain D segments, J segments,

and $\gamma 1$ constant regions together with representatives of all 6 V_H families (although this fragment is identified as 670 kb fragment from HeLa cells by Berman et al. (1988), supra., we have found it to be an 830 kb fragment from human placental and

5   sperm DNA). Those fractions containing this NotI fragment (see Fig. 4) are ligated into the NotI cloning site of the vector pYACNN as described (McCormick, M. et al. (1990), <u>Technique</u> 2, 65-71). Plasmid pYACNN is prepared by digestion of pYACneo (Clontech) with EcoRI and ligation in the presence

10 of the oligonucleotide 5' - AAT TGC GGC CGC - 3'.

YAC clones containing the heavy chain NotI fragment are isolated as described by Traver et al. (1989), <u>Proc. Natl. Acad. Sci. USA</u>, <u>86</u>, 5898-5902. The cloned NotI insert is isolated from high molecular weight yeast DNA by pulse field

15 gel electrophoresis as described by M. Finney, op. cit. The DNA is condensed by the addition of 1 mM spermine and microinjected directly into the nucleus of single cell embryos previously described. Alternatively, the DNA is isolated by pulsed field gel electrophoresis and introduced into ES cells

20 by lipofection (Gnirke et al. (1991), <u>EMBO J.</u>, <u>10</u>, 1629-1634), or the YAC is introduced into ES cells by spheroplast fusion.

EXAMPLE 20

25 <u>Discontinuous Genomic Heavy Chain Ig Transgene</u>

An 85 kb SpeI fragment of human genomic DNA, containing $V_H 6$, D segments, J segments, the μ constant region and part of the γ constant region (see Fig. 4), has been isolated by YAC cloning essentially as described in Example 1.

30 A YAC carrying a fragment from the germline variable region, such as a 570 kb NotI fragment upstream of the 670-830 kb NotI fragment described above containing multiple copies of V_1 through V_5, is isolated as described. (Berman et al. (1988), supra. detected two 570 kb NotI fragments, each containing

35 multiple V segments.) The two fragments are coinjected into the nucleus of a mouse single cell embryo as described in Example 1.

Typically, coinjection of two different DNA fragments
result in the integration of both fragments at the same
insertion site within the chromosome.   Therefore,
approximately 50% of the resulting transgenic animals that
5   contain at least one copy of each of the two fragments will
have the V segment fragment inserted upstream of the constant
region containing fragment.   Of these animals, about 50% will
carry out V to DJ joining by DNA inversion and about 50% by
deletion, depending on the orientation of the 570 kb NotI
10   fragment relative to the position of the 85 kb SpeI fragment.
DNA is isolated from resultant transgenic animals and those
animals found to be containing both transgenes by Southern blot
hybridization (specifically, those animals containing both
multiple human V segments and human constant region genes) are
15   tested for their ability to express human immunoglobulin
molecules in accordance with standard techniques.

EXAMPLE 21

Joining Overlapping YAC Fragments

20   Two YACs carrying a region of overlap are joined in
yeast by meiotic recombination as described by Silverman et al.
(1990), Proc. Natl. Acad. Sci. USA, 87, 9913-9917, to derive a
single, large YAC carrying sequences from both smaller YACs.
The two YACs are aligned with respect to the arms, such that
25   the joined YAC will contain one centromeric vector arm and one
non-centromeric vector arm.   If necessary, the insert is
recloned in the vector using unique restriction sites at the
ends of the insert.   If the insert is not a unique restriction
fragment, unique sites are inserted into the vector arms by
30   oligonucleotide transformation of yeast, as described by
Guthrie and Fink, op. cit.   To join YACs carrying noncontiguous
sequences which do not overlap, an overlap is created as
follows.   The 3' terminal region of the 5' YAC and the 5'
terminal region of the 3' YAC are subcloned, joined in vitro to
35   create a junction fragment, and reintroduced into one or both
YACs by homologous recombination (Guthrie and Fink, op cit).
The two YACs are then meiotically recombined as described by

Silverman et al., op cit). The joined YAC is introduced into
mice, e.g., as in Example 1.

                        EXAMPLE 22

5   Genomic κ Light Chain Human Ig Transgene

        A map of the human κ light chain has been described in
Lorenz, W. et al. (1987), Nucl. Acids Res., 15, 9667-9677 and
is depicted in Fig. 11. A 450 kb XhoI to NotI fragment that
includes all of Cκ, the 3' enhancer, all J segments, and at
10   least five different V segments (a), or a 750kb MluI to NotI
fragment that includes all of the above plus at least 20 more V
segments (b) is isolated and introduced into zygotes or ES
cells as described in Example 1.

15   EXAMPLE 23

Genomic κ Light Chain Human Ig Transgene Formed by In Vivo
Homologous Recombination

        The 750kb MluI to NotI fragment is digested with
BssHII to produce a fragment of about 400 kb (c). The 450 kb
20   XhoI to NotI fragment (a) plus the approximately 400 kb MluI to
BssHII fragment (c) have sequence overlap defined by the
BssHII and XhoI restriction sites shown in Fig. 11. Homologous
recombination of these two fragments upon microinjection of a
mouse zygote results in a transgene containing at least an
25   additional 15-20 V segments over that found in the 450 kb
XhoI/NotI fragment (Example 22).

                        EXAMPLE 24

Identification of functionally rearranged variable region
30   sequences in transgenic B cells

        An antigen of interest is used to immunize (see Harlow
and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor,
New York (1988)) a mouse with the following genetic traits:
homozygosity at the endogenous having chain locus for a
35   deletion of J_H (Examples 9 and 12); hemizygous for a single
copy of unrearranged human heavy chain minilocus transgene
(examples 5 and 14); and hemizygous for a single copy of a

108

rearranged human kappa light chain transgene (Examples 7 and 16).

Following the schedule of immunization, the spleen is removed, and spleen cells used to generate hybridomas. Cells

5   from an individual hybridoma clone that secretes antibodies reactive with the antigen of interest are used to prepare genomic DNA. A sample of the genomic DNA is digested with several different restriction enzymes that recognize unique six base pair sequences, and fractionated on an agarose gel.

10 Southern blot hybridization is used to identify two DNA fragments in the 2-10 kb range, one of which contains the single copy of the rearranged human heavy chain VDJ sequences and one of which contains the single copy of the rearranged human light chain VJ sequence. These two fragments are size

15 fractionated on agarose gel and cloned directly into pUC18. The cloned inserts are then subcloned respectively into heavy and light chain expression cassettes that contain constant region sequences.

The plasmid clone pγe1 (Example 14) is used as a heavy

20 chain expression cassette and rearranged VDJ sequences are cloned into the XhoI site. The plasmid clone pCK1 is used as a light chain expression cassette and rearranged VJ sequences are cloned into the XhoI site. The resulting clones are used together to transfect SP_0 cells to produce antibodies that

25 react with the antigen of interest (M.S. Co. et al. (1991) Proc. Natl. Acad. Sci. USA 88:2869).

Alternatively, mRNA is isolated from the cloned hybridoma cells described above, and used to synthesize cDNA. The expressed human heavy and light chain VDJ and VJ sequence

30 are then amplified by PCR and cloned (J.W. Larrich et al. (1989) Biol. Technology, 7:934-938). After the nucleotide sequence of these clones has been determined, oligonucleotides are synthesized that encode the same polypeptides, and synthetic expression vectors generated as described by C. Queen

35 et al. (1989) Proc. Natl. Acad. Sci. USA., 84:5454-5458.

The foregoing description of the preferred embodiments of the present invention has been presented for purposes of illustration and description. They are not intended to be

exhaustive or to limit the invention to the precise form disclosed, and many modifications and variations are possible in light of the above teaching.

5   All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Such modifications and variations which may be apparent to a person skilled in the art are intended to be

10   within the scope of this invention.

WHAT IS CLAIMED IS:

1.   An isolated immunoglobulin heavy-chain transgene
comprising DNA encoding at least one variable gene segment, one
diversity gene segment, one joining gene segment, and one
constant region gene segment, wherein each of said gene
segments is derived from DNA corresponding to immunoglobulin
heavy chain gene segments from the same species or an
individual of said species and wherein the segments are capable
of undergoing gene rearrangement in vivo to form a rearranged
gene encoding a heavy chain polypeptide.

2.   An immunoglobulin heavy-chain transgene of Claim
1 wherein the length of said transgene is less than the length
of the corresponding genomic DNA containing all of the gene
segments contained on said heavy-chain transgene.

3.   The transgene of Claim 1 wherein said at least
one constant region gene segment comprises at least two
constant region gene segments.

4.   The transgene of Claim 3 wherein said one
constant region gene segments comprises a μ and a γ constant
region gene segment.

5.   The transgene of Claim 1 wherein said at least
one constant region gene segment comprises a γ constant region
gene segment.

6.   The transgene of Claim 1 wherein said at least
one variable gene segment comprises DNA corresponding to a
first functional D-proximal variable region gene segment of
said species or said individual.

7.   The transgene of Claim 1 wherein the relative
positions of each of said gene segments is the same as the
relative position of the corresponding gene segments in the
genome of said species.

8. The transgene of Claim 1 wherein said species is human.

5   9. The transgene of Claim 1 wherein said transgene is unrearranged and capable of undergoing rearrangement to generate V region diversity, when introduced into a B-cell of a non-human transgenic animal.

10   10. An isolated immunoglobulin light-chain transgene comprising DNA encoding at least one variable gene segment, one joining gene segment, and one constant region gene segment, wherein each of said gene segments is derived from genomic DNA corresponding to immunoglobulin light chain gene segments from 15 the same species or an individual of said species and wherein the segments are capable of undergoing gene rearrangement in vivo to form a rearranged gene encoding a light chain polypeptide.

20   11. An immunoglobulin light-chain transgene of Claim 9 wherein the length of said transgene is less than the length of the corresponding genomic DNA containing all of the gene segments contained on said light-chain transgene.

25   12. The transgene of Claim 11 wherein said at least one variable region gene segment comprises DNA corresponding to a first functional J-proximal variable gene segment of said species or said individual.

30   13. The transgene of Claim 11 wherein the relative positions of said gene segments is the same as the relative positions of the corresponding gene segments in the genome of said species or said individual.

35   14. The transgene of Claim 9 wherein said species or individual is human.

15. The transgene of Claim 10 wherein said transgene is unrearranged and capable of undergoing rearrangement to generate V region diversity, when introduced into a B-cell of a non-human transgenic animal.

16. A transgenic non-human animal containing heterologous immunoglobulin heavy and light chain transgenes in the germline of said animal.

17. The transgenic non-human animal of Claim 16 wherein said heavy and said light chain transgenes are rearranged.

18. The transgenic non-human animal of Claim 16 wherein said heavy and said light chain transgenes are unrearranged.

19. The transgenic non-human animal of Claim 16 wherein one of said heavy and said light chain transgenes is rearranged and the other of said heavy and said light transgenes is unrearranged.

20. The transgenic non-human animal of Claim 19 wherein said heavy chain transgene is unrearranged and said light chain transgene is rearranged.

21. The transgenic non-human animal of Claim 16 wherein said transgenes are functionally rearranged in B-cells of said animal.

22. The transgenic non-human animal of Claim 21 wherein said B-cells produce a heterologous antibody.

23. The transgenic non-human animal of Claim 16 wherein said heterologous heavy and light chain genes are human.

113

24. The transgenic non-human animal of Claim 16 wherein said non-human animal is a rodent.

5   25. A transgenic non-human animal capable of producing a heterologous antibody in at least one cell of said transgenic animal containing an immunoglobulin heavy-chain transgene and an immunoglobulin light-chain transgene, wherein said heavy chain transgene comprises DNA encoding at least one variable gene segment, one diversity gene segment, one joining

10   gene segment, and one constant region gene segment, and said immunoglobulin light-chain transgene comprises DNA encoding at least one variable gene segment, one joining gene segment, and one constant region gene segment, wherein each of said gene segments of said heavy and light chain transgenes is isolated

15   from or corresponds to DNA encoding immunoglobulin heavy and light chain gene segments from a species not consisting of said non-human animal or an individual of said species.

26. The transgenic animal of Claim 25 wherein said

20   transgenic non-human animal is a rodent.

27. The transgenic non-human animal of Claim 25 wherein said transgenes encode human antibody gene segments.

25   28. The transgenic non-human animal of claim 25 wherein at least one of the endogenous immunoglobulin loci of said transgenic animal is functionally disrupted.

29. The transgenic non-human animal of Claim 28

30   wherein the functional disruption of said endogenous loci is produced by disrupting endogenous immunoglobulin gene segments encoding heavy or light immunoglobulin chains, said gene segments being selected from the group consisting of diversity, joining and constant gene segments.

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30. The transgenic non-human animal of Claim 29 wherein said endogenous immunoglobulin gene segments are

114

joining region gene segments encoding said heavy and light
immunoglobulin chains.

5   31.   The transgenic non-human animal of Claim 29
wherein said disruption is by deletion of substantially all of
said selected gene segments.

         32.   The transgenic non-human animal of Claim 25
wherein said heavy and said light chain transgenes are
10   unrearranged.

         33.   The transgenic non-human animal of Claim 25
wherein said heavy and said light chain transgenes are
rearranged.
15

         34.   The transgenic non-human animal of Claim 25
wherein one of said heavy and said light chain transgenes is
rearranged and the other of said heavy and said light chain
transgenes is unrearranged.
20

         35.   The transgenic non-human animal of Claim 34
wherein said heavy chain transgenes is unrearranged and said
light chain transgenes is rearranged.

25   36.   A non-human B-cell derived from a transgenic non-
human animal, said B-cell being capable of producing a
heterologous antibody.

         37.   The non-human B-cell of Claim 36 wherein said B-
30   cell contains a functionally rearranged heterologous heavy
chain immunoglobulin transgene and a functionally rearranged
heterologous light chain immunoglobulin transgene.

         38.   The non-human B-cell of Claim 37 wherein each of
35   said functionally rearranged heavy and light chain
immunoglobulin transgenes are of human origin.

39.   The non-human B-cell of Claim 36 wherein said
B-cell does not produce a homologous antibody.

40.   A hybridoma comprising a myeloma cell fused to a
non-human B-cell derived from a transgenic non-human animal,
said hybridoma capable of producing a monoclonal antibody
heterologous to said B-cell.

41.   The hybridoma of Claim 40 wherein genomic
material of said hybridoma derived from said non-human B-cell
contains a functionally rearranged heavy chain immunoglobulin
transgene and a functionally rearranged light chain
immunoglobulin transgene, each of said functionally rearranged
transgenes being heterologous to said B-cell.

42.   The hybridoma of Claim 41 wherein said transgenes
are of human origin.

43.   The hybridoma of Claim 40 wherein said monoclonal
antibody is a human antibody.

44.   The hybridoma of Claim 40 wherein said B-cell is
of rodent origin.

45.   The hybridoma of Claim 40 wherein said myeloma
cell is of murine origin.

46.   A method of producing a transgenic non-human
animal wherein at least one of the endogenous immunoglobulin
gene loci of said animal has been functionally disrupted, said
method comprising the steps of:
contacting at least one embryonic stem cell of a
non-human animal with a transgene targeting the functional
disruption of a family of gene segments encoding an endogenous
heavy or light immunoglobulin chain, said family of gene
segments being selected from the group consisting of functional
endogenous diversity, joining and constant gene segment
families; and

selecting at least one embryonic stem cell wherein
said transgene has integrated into the genome of said non-human
animal by homologous recombination.

5   47.   The method of Claim 46 wherein said transgene
comprises a positive-negative selection vector.

           48.   The method of Claim 46 wherein said functional
disruption comprises the deletion of all or part of said family
10   of gene segments.

           49.   The method of Claim 48 wherein said deletion is
of the family of joining gene segments.

15   50.   The method of Claim 46 wherein said functional
disruption comprises the introduction of a transcription or
translation stop sequence.

           51.   The method of Claim 46 wherein said functional
20   disruption is of a constant region gene segment.

           52.   The method of Claim 51 wherein said gene segment
is selected from the group consisting of the μ heavy chain
constant region gene segment, the κ light chain constant region
25   gene segment and the group of functional λ light chain
construct region gene segments.

           53.   A method for producing a heterologous antibody in
a transgenic non-human animal having at least one B-cell
30   containing at least one rearranged heterologous immunoglobulin
heavy chain transgene and at least one rearranged heterologous
immunoglobulin light chain transgene, said antibody being
capable of binding an antigen, said method comprising the step
of:
35   contacting said transgenic animal with said antigen
to induce the production of said heterologous antibody.

54.   The method of Claim 53 wherein said contacting causes somatic mutation of at least one of said transgenes.

55.   The method of Claim 53 further comprising the step of immortalizing at least one of the B-cells producing said heterologous antibody to provide a source of monoclonal antibody corresponding to said heterologous antibody.

56.   The method of Claim 55 wherein said immortalizing is by fusion of said B-cell with a myeloma cell line to form a hybridoma.

57.   A monoclonal antibody made according to the method of Claim 55.

58.   The monoclonal antibody of Claim 57 wherein said heavy and light chain transgenes comprise human immunoglobulin gene segments and said antibody is a human antibody.

59.   A method for producing a first heterologous antibody in a transgenic non-human animal having at least one B-cell containing at least one rearranged heterologous immunoglobulin heavy chain transgene and at least one rearranged heterologous immunoglobulin light chain transgene, wherein said first heterologous antibody is capable of binding a first antigen and said heavy and light transgenes are capable of undergoing somatic mutation in response to a known second antigen to produce a second heterologous antibody, said method comprising the step of:

contacting said transgenic animal with at least said first antigen to induce the somatic mutation of at least one of said heavy or light chain transgenes to produce a B-cell capable of producing said first heterologous antibody.

60.   The method of Claim 59 wherein said contacting comprises the sequential or simultaneous contacting of said first antigen and said second known antigen to produce first

and second B-cells capable of producing said first and said
second heterologous antibodies respectively.

        61.   The method of Claim 59 further comprising the
step of immortalizing at least one of said B-cells producing
said first heterologous antibody to provide a source of
monoclonal antibody corresponding to said first heterologous
antibody.

        62.   The method of Claim 61 wherein said immortalizing
is by fusion of said B-cell with a myeloma cell line to form a
hybridoma.

        63.   A monoclonal antibody made according to the
method of Claim 61.

        64.   The monoclonal antibody of Claim 63 wherein said
heavy and light chain rearranged transgenes comprise human
immunoglobulin gene segments and said antibody is a human
antibody.

        65.   A method for producing a synthetic immunoglobulin
V segment repertoire comprising the steps of:
                (a)   generating a population of immunoglobulin V
segment DNAS, each of said V segment DNAs encoding an
immunoglobulin V segment and containing at each end a first
cleavage recognition site of a first restriction endonuclease;
and
                (b)   concatenating said population of
immunoglobulin V segment DNAs to form a synthetic
immunoglobulin V segment repertoire.

        66.   The method of Claim 65 wherein said population of
V segment DNAs is derived from genomic DNA and said generating
is by PCR amplification using P1 and P2 primers, said P1 primer
comprising a mixture of primers encoding, from 5' to 3', said
cleavage recognition site and sequences capable of hybridizing
to one strand of the C-terminal portion of a multiplicity of

immunoglobulin V segments in said genomic DNA, and said P2
primer comprising a mixture of primers encoding, from 5' to 3',
said cleavage recognition site and sequences capable of
hybridizing to the complementary strand of the N-terminal
5   portion of said multiplicity of immunoglobulin V segments in
said genomic DNA.

67.   The method of Claim 65 wherein said population of
immunoglobulin V segment DNAs is derived from B-cell mRNA and
10   said generating comprises the steps of:
(i)   priming synthesis of single stranded cDNA
from said mRNA with primer P1 comprising a mixture of primers
encoding, from 5' to 3', said cleavage recognition site and
sequences capable of hybridizing to the coding strand of the
15   C-terminal portion of a multiplicity of immunoglobulin V
segments in the genomic DNA from which said mRNA is
transcribed;
(ii) priming synthesis of double stranded cDNA
from said single stranded cDNA with primer P2 comprising a
20   mixture of primers encoding, from 5' to 3', said cleavage
recognition site and sequences capable of hybridizing to the
antisense strand of the N-terminal portion of said multiplicity
of immunoglobulin V segments in said genomic DNA.

25   68.   The method of Claim 67 wherein each of said
immunoglobulin V segment DNAs contains a first cleavage
recognition site at one end of said V segment DNA and a second
different cleavage recognition site at the other end of said V
segment DNA and said method further comprises the step of:
30   amplifying said double stranded cDNA using P3 and P4
primers, said P3 primer comprising DNA encoding said first
cleavage recognition site and said P4 primer comprising DNA
encoding said second cleavage recognition site.

35   69.   The method of Claim 65 wherein said
immunoglobulin V segments comprise DNA encoding the first
signal sequence exon and second exon of more than one
immunoglobulin V segment encoded by genomic DNA.

120

70. The method of Claim 65 wherein said population
immunoglobulin V segment DNAs comprise DNA encoding the second
exon of more than one immunoglobulin V segment encoded by
5 genomic DNA.

71. The method of Claim 70 wherein each of the
members of said population is ligated into an expression vector
to form an expression cassette comprising, in order, a second
10 cleavage site of a second restriction endonuclease, an
immunoglobulin promoter, an immunoglobulin secretion signal
sequence, a third cleavage recognition site of a third
restriction endonuclease, a recombination signal sequence and a
fourth cleavage recognition site of a fourth restriction
15 endonuclease, said ligation being into said third cleavage
recognition site to form said expression cassette between said
second and said fourth cleavage recognition site.

72. The method of Claim 71 wherein said concatenation
20 is of the expression cassette by digesting said expression
vector with said second and said fourth restriction
endonucleases.

73. The synthetic V segment repertoire made according
25 to the method of Claim 65.

74. A method for producing an expressible V segment
comprising the steps of:
        (a)   generating at least one V segment DNA
30 encoding the second exon of a V segment and containing at each
end thereof a first cleavage recognition site of a first
restriction endonuclease; and
        (b)   ligating said V segment DNA into an
expression vector, said expression vector comprising, in order,
35 an immunoglobulin promoter, an immunoglobulin secretion signal
sequence, a second cleavage recognition site of a second
restriction endonuclease capable of ligating said V segment DNA

when said vector is cleaved with said second restriction
endonuclease and a recombination sequence.

75.   An immunoglobulin (Ig) heavy chain minilocus
transgene construct formed from a plurality of DNA fragments,
the construct comprising DNA sequences that encode human
variable (V), diversity (D), joining (J) and constant regions
of a human Ig protein, which sequences are operably linked to
transcription regulatory sequences and capable of undergoing
gene rearrangement in vivo to produce a rearranged gene
encoding a human heavy chain polypeptide; wherein each of at
least three of the DNA fragments comprises:
        a V region sequence,
        a D region sequence,
        a J and constant region sequence,
        a D and J and constant region sequence, or
        a constant region sequence;
wherein all of the Ig protein coding sequences are
substantially homologous to human gene sequences.

76.   The minilocus transgene construct of claim 75
wherein the minilocus transgene construct is about 75 kb in
length.

77.   The minilocus transgene construct of claim 75
wherein the constant region gene sequences encode two different
immunoglobulin isotypes.

78.   The minilocus transgene construct of claim 77
wherein the constant region gene sequences are capable of
undergoing switch recombination.

79.   The minilocus transgene construct of claim 77
wherein the isotypes are mu and gamma.

80.   The minilocus transgene construct of claim 75
wherein constant region gene sequences encode a heavy chain
gamma isotype.

81. The minilocus transgene construct of claim 75 wherein the Ig coding sequences are from a single individual.

82. An Ig minilocus transgene construction of claim 75 wherein the constant region sequence comprises a μ constant region 5' from a second constant region.

83. An Ig minilocus transgene construction of claim 82, wherein the construct further comprises a switch donor region 5' from the μ constant region and a switch acceptor region between the μ constant region and the second constant region, which switch regions are operably linked to effect switching in vivo.

84. An Ig minilocus transgene construction of claim 83, wherein the switch donor region is a human μ switch region.

85. An Ig minilocus transgene construction of claim 83, wherein the switch acceptor region is a human γ_1 switch region.

86. An Ig minilocus transgene construction of claim 83, wherein the second constant region is a γ_1 constant region.

87. An immunoglobulin (Ig) light chain minilocus transgene construct formed from a plurality of DNA fragments, the construct comprising DNA sequences that encode human variable (V), joining (J) and constant regions of a human Ig protein, which sequences are operably linked to transcription regulating sequences and are capable of undergoing gene rearrangement in vivo to produce a rearranged gene encoding a human light chain polypeptide; wherein each of at least three of the DNA fragments comprises:
        a V region sequence,
        a J and constant region sequence, or
        a constant region sequence;

wherein all of the Ig protein coding sequences are substantially homologous to human gene sequences.

88.   The minilocus transgene construct of claim 87 wherein the gene sequences are from a single individual.

89.   The minilocus transgene construct of claim 87 wherein the minilocus transgene construct consists of about 50 kb.

90.   The minilocus transgene construct of claim 87 wherein one variable gene is operably joined to the joining gene sequence.

91.   The minilocus transgene construct of claim 87 wherein the relative position of each region is the same as that of a corresponding region in a germline light chain gene.

92.   A yeast artificial chromosome (YAC) containing an immunoglobulin gene insert comprising:
        an immunoglobulin variable gene sequence, a joining gene sequence and a constant region sequence which are capable of effecting rearrangement in vivo to form a rearranged gene of the sequences, which gene encodes an immunoglobulin polypeptide.

93.   The YAC of claim 92 wherein the chromosome further comprises a diversity gene sequence.

94.   The YAC of claim 92 wherein the chromosome further comprises a switch donor region and a switch acceptor region which are operably linked to effect isotype switching in vivo.

95.   The YAC of claim 92 wherein the gene sequences are from the same individual.

96.   The YAC of claim 92 wherein the insert is about 85 kb.

124

97. The YAC of claim 92 further comprising transcription regulatory sequences operably linked to the insert.

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98. A method of producing a modified genome in a transgenic non-human mammal, the method comprising:

condensing two yeast artificial chromosomes having overlapping sequences, which chromosomes together encode a

10   functional immunoglobulin locus; and

inserting the yeast artificial chromosomes into the mammal genome.

99. The method of claim 98 wherein the yeast

15   artificial chromosome is condensed with polyamines.

100. The method of claim 98, wherein the inserting step is by transfecting or lipofecting.

20   101. The method of claim 98 wherein the yeast artificial chromosome is integrated into the genome.

102. A method of inactivating an endogenous immunoglobulin gene in a non-human mammal, the method

25   comprising introducing into the genome of the mammal a DNA fragment comprising a nucleotide sequence homologous to the endogenous immunoglobulin gene, whereby the nucleotide fragment incorporates into the genome by homologous recombination and thereby disrupts the endogenous gene.

30

103. The method of claim 102 wherein the step of introducing the DNA fragment into the genome is carried out by transforming an embryonic stem cell.

35   104. The method of claim 102 wherein the disruption comprises deletion of joining gene segments.

105. The method of claim 102 wherein the disruption comprises deletion of an enhancer or of a kappa constant region.

5   106. A non-human mammal produced by the method of claim 102.

107. An isolated immunoglobulin chain minilocus transgene construct of claim 75 or 87, wherein the V region

10   sequence comprises VH251.

108. A transgenic non-human mammal capable of producing a rearranged human immunoglobulin and having an endogenous immunoglobulin gene with a heterologous nucleotide

15   sequence insert.

109. A mammal of claim 108 wherein the heterologous nucleotide sequence decreases expression of endogenous immunoglobulin polypeptides.

20

110. A mammal of claim 108 wherein the heterologous nucleotide sequence decreases expression of endogenous μ heavy chain polypeptides.

25   111. A mammal of claim 108 wherein the heterologous nucleotide sequence decreases expression of endogenous light chain polypeptides.

112. A mammal of claim 111 wherein the light chain

30   polypeptides are kappa or lambda.

113. A mammal of claim 108 wherein the heterologous nucleotide sequence introduces a transcription or translation stop sequence.

35

114. A mammal of claim 108 wherein the mammal is a mouse.

115. A mammal of claim 108 wherein the immunoglobulin is expressed from an immunoglobulin minilocus transgene construct having a variable gene segment, a joining gene segment, and a constant region gene segment.

5

116. A mammal of claim 115 wherein the minilocus transgene construct is a heavy chain gene.

117. A mammal of claim 108 comprising a rearranged

10   immunoglobulin transgene construct.

118. A mammal of claim 117 wherein the rearranged transgene construct encodes a kappa light chain.

15   119. A mammal of claim 117 wherein the rearranged transgene construct encodes a heavy chain.

120. A method for producing an immunoglobulin from a transgenic non-human mammal, the mammal having a rearranged

20   immunoglobulin transgene construct that recognizes a selected antigen, the method comprising:
            immunizing the transgenic mammal with the selected antigen; and
            screening for B cells from the transgenic mammal that

25   produce immunoglobulins that bind the selected antigen.

121. The method of claim 120 wherein the step of screening includes immortalizing B cells isolated from the transgenic mammal.

30

122. An immortalized cell produced according to the method of claim 121, wherein the cell is a hybridoma formed by fusing the selected B cell with a myeloma cell.

35   123. A monoclonal antibody produced by the hybridoma of claim 120.

124. A method according to claim 120, further comprising the steps of:

immunizing the transgenic animal with a second antigen prior to the screening step; and

screening for B cells that produce immunoglobulins that bind the second antigen.

125. The method of claim 120, wherein one of the antigens is a human immunoglobulin.

126. The method of claim 120, wherein the mouse comprises T-cell receptor transgenes and one of the antigens is a human T-cell receptor.

127. A transgenic mouse having a genome comprising:

(i)   a heavy chain human transgene comprising a variable gene sequence, a diversity gene sequence, a joining gene sequence, and two constant region sequences operably linked to a switch donor sequence and a switch acceptor sequence;

(ii) a light chain transgene comprising a variable gene sequence, a joining gene sequence and a constant gene sequence; and

(iii) transcription regulating sequences operably linked to each transgene; wherein the gene sequences and regulatory sequences effect production of developmentally regulated diversity in vivo to form a plurality of human immunoglobulins.

128. The transgenic mouse of claim 127 which produces about a mg of human immunoglobulins per ml serum

129. The transgenic mouse of claim 127 wherein greater than about 10% of the human immunoglobulins are IgG.

130. A transgenic mouse of claim 127 wherein the human immunoglobulins exhibit an affinity for preselected antigens of greater than about $10^{-7}\underline{M}^{-1}$.

131. The transgenic mouse of claim 127 which mounts an immune response when immunized against foreign antigens, which immune response comprises producing human immunoglobulins that

5   are capable of binding to at least about 10% of antigens bound by endogenous immunoglobulins of a non-transgenic mouse immunized with the antigens.

132. The transgenic mouse of claim 131 which is

10   capable of producing greater than about 1000 different human immunoglobulins.

133. The transgenic mouse of claim 131 which is capable of producing greater than about 1000 different human

15   IgG immunoglobulins.

134. A method of producing human immunoglobulins comprising immortalizing B cells from a transgenic mouse of claim 131 immunized with an antigen; and growing selected

20   immortalized B-cells secreting immunoglobulins reactive with the antigen.

135. A human immunoglobulin (Ig) produced according to claim 134 which Ig is isolated with respect to human proteins

25   natively associated with human immunoglobulins.

136. A human immunoglobulin of claim 135 which has an affinity for the antigen of greater than $10^{-7}\underline{M}^{-1}$.

30   137. A transgenic mouse having a genome comprising a human immunoglobulin heavy chain minilocus transgene, the transgene being capable of rearrangement and switching in the mouse, whereby two or more rearranged human heavy chain isotypes are produced.

35

138. The transgenic mouse of claim 137, wherein the isotypes are μ and γ.

139. An isolated human monoclonal antibody produced by a mouse of claim 137.

140. A human immunoglobulin specifically reactive with
5   a lymphoid cell subset.

141. A human immunoglobulin of claim 140, wherein the subset is a human T-cell subset.

10   142. A human immunoglobulin of claim 141, wherein the subset is autoreactive T-cells.

143. A plasmid vector useful in cloning immunoglobulin DNA fragments, the vector comprising
15   (i)   an origin of replication;
             (ii)   a copy control region;
             (iii) a cloning site flanked by rare restriction
enzyme sites;
             (iv)   a transcriptional terminator located
20   downstream of a plasmid-derived promoter and upstream of the cloning site, whereby a transcript originating at the promoter is terminated upstream of the cloning site.

144. A vector according to claim 143, wherein the rare
25   restriction enzyme sites are selected from NotI, SfiI, and PacI.

145. A vector according to claim 143, selected from the group consisting of pGP1b, pGP1c, pGP1d, pGP1f and pGPe.

30

146. A composition consisting essentially of a human VDJ rearranged gene fragment isolated from a B cell developed within a transgenic mouse of claim 16, 25, 108, 127, or 137.

35   147. A method for producing a human immunoglobulin from a transgenic rodent, the rodent having a genome comprising germline copies of human heavy and light chain immunoglobulin transgenes, the method comprising:

immunizing the rodent with an antigen; and

screening for B cells from the rodent that produce
immunoglobulins that bind the antigen.

5   148. The method of claim 147, wherein the light chain
transgene is rearranged in the germline.

149. The method of claim 148, wherein the heavy chain
transgene is unrearranged in the rodent germline.

10

150. An isolated human immunoglobulin produced
according to the method of claims 40, 53, 59, 120, or 147.
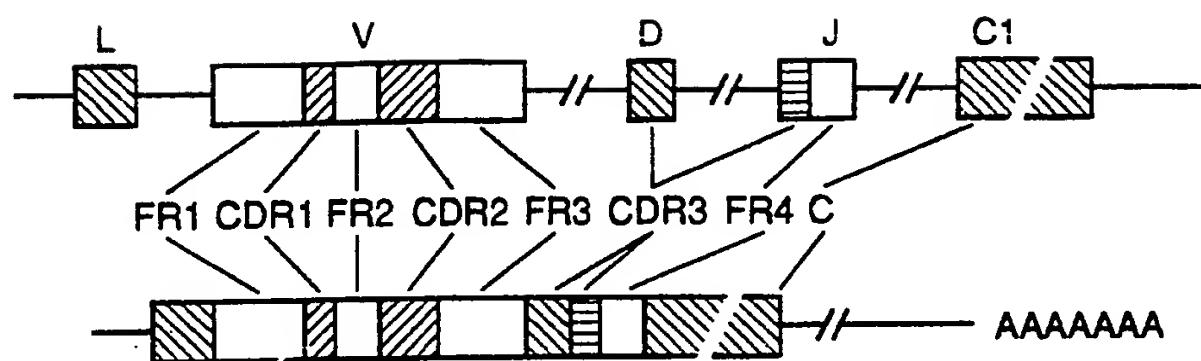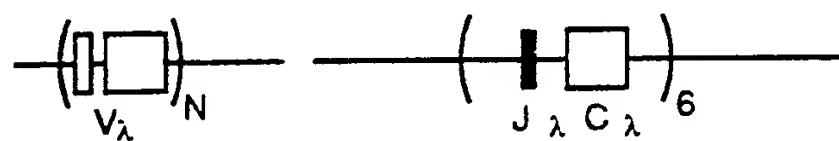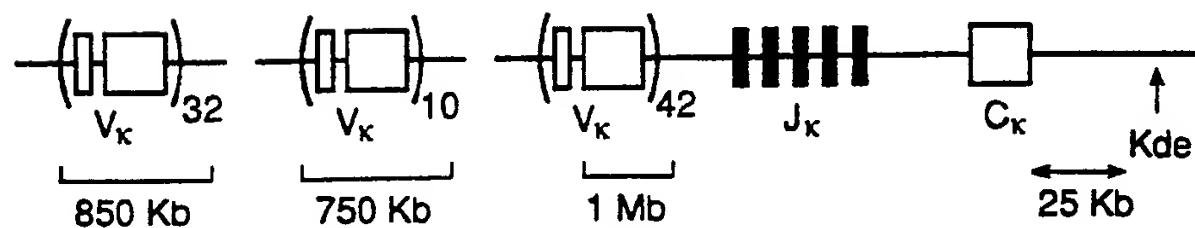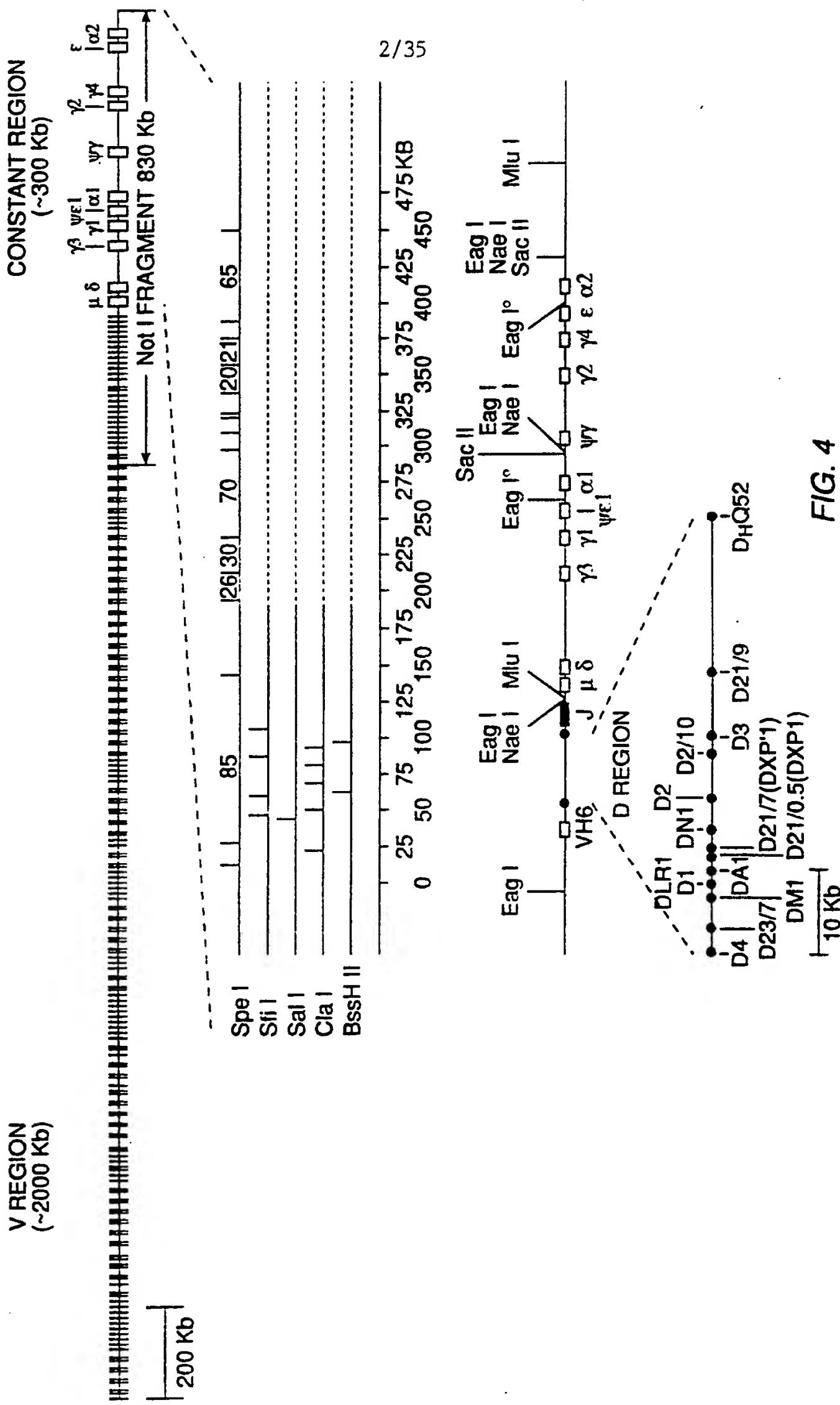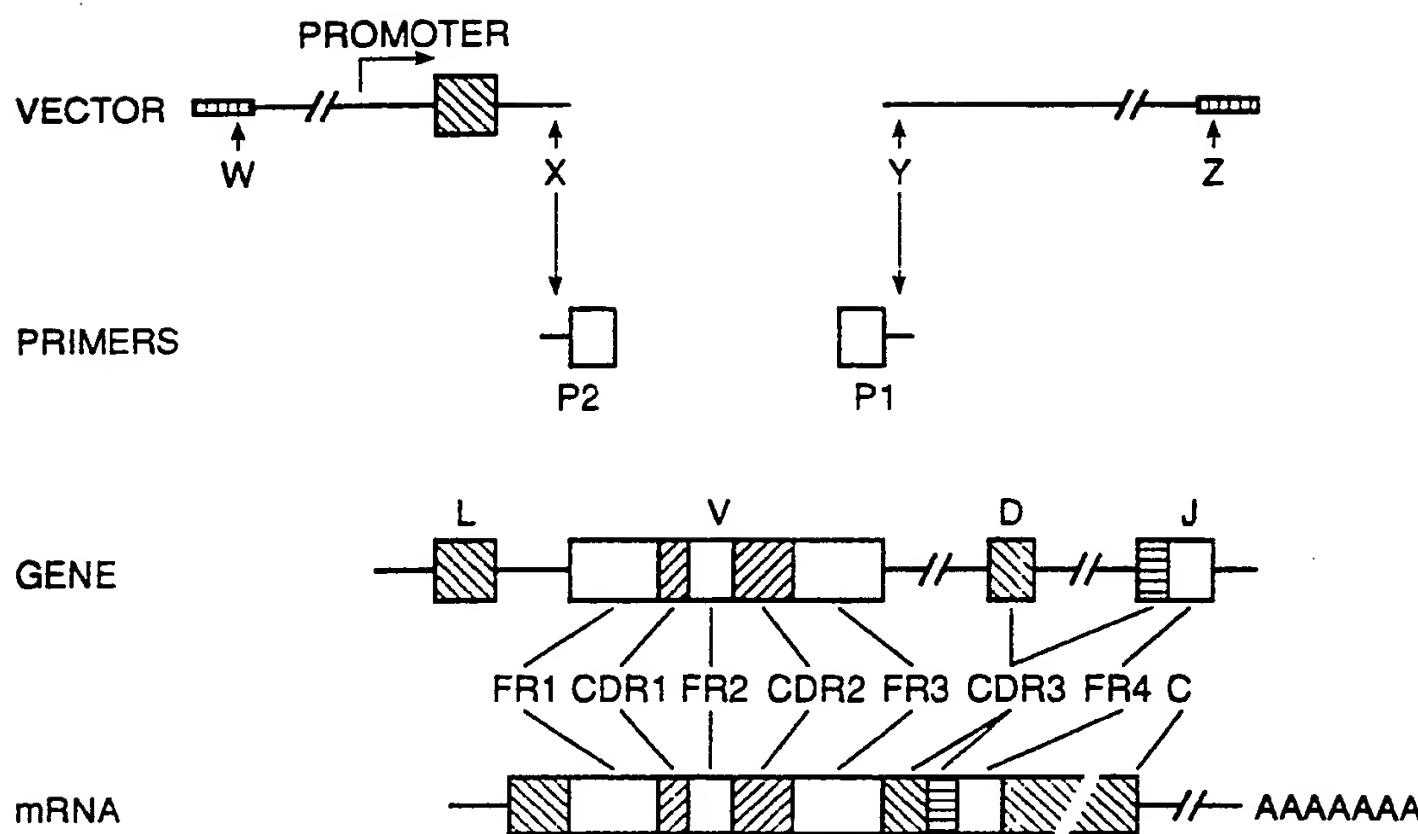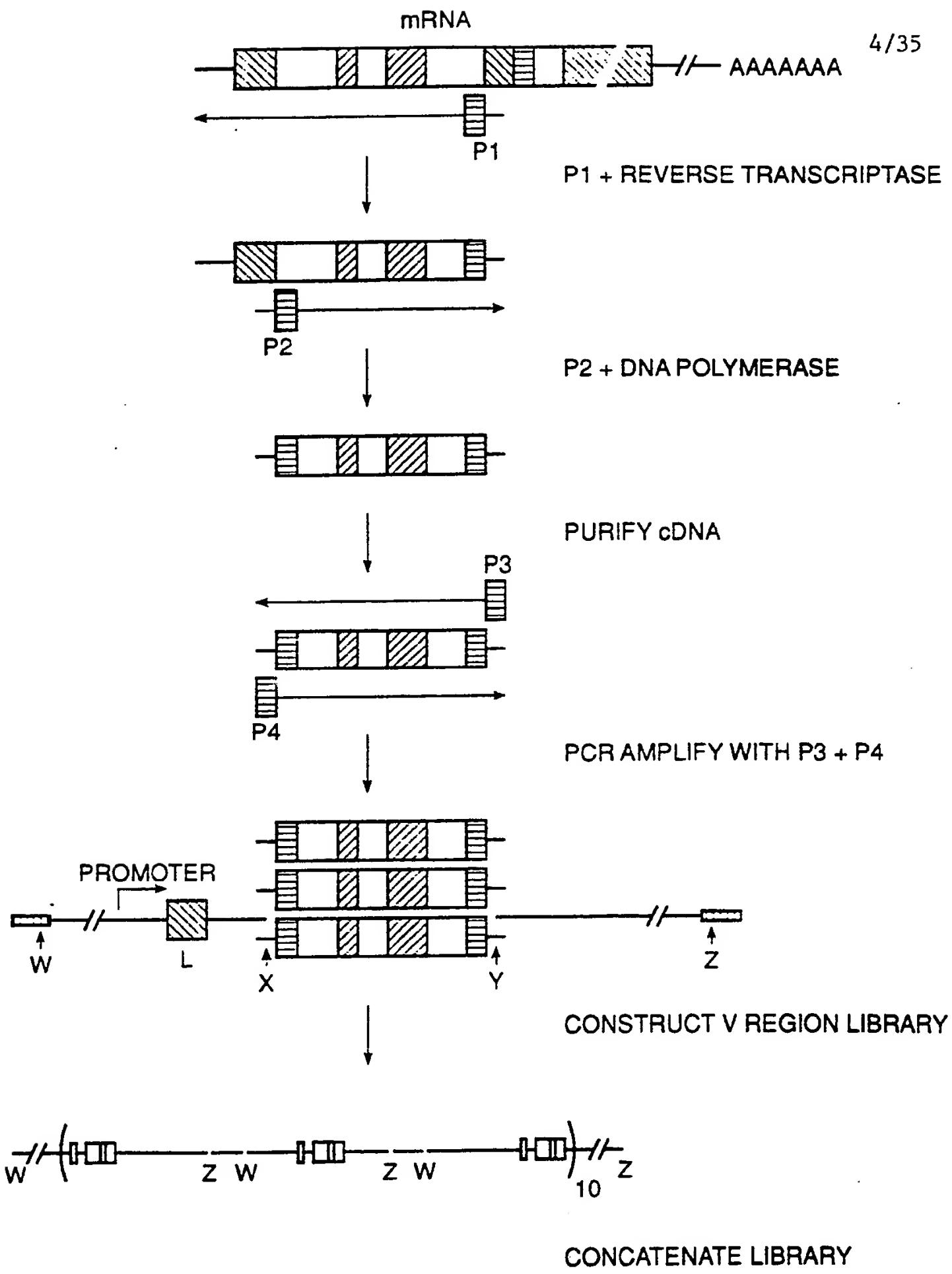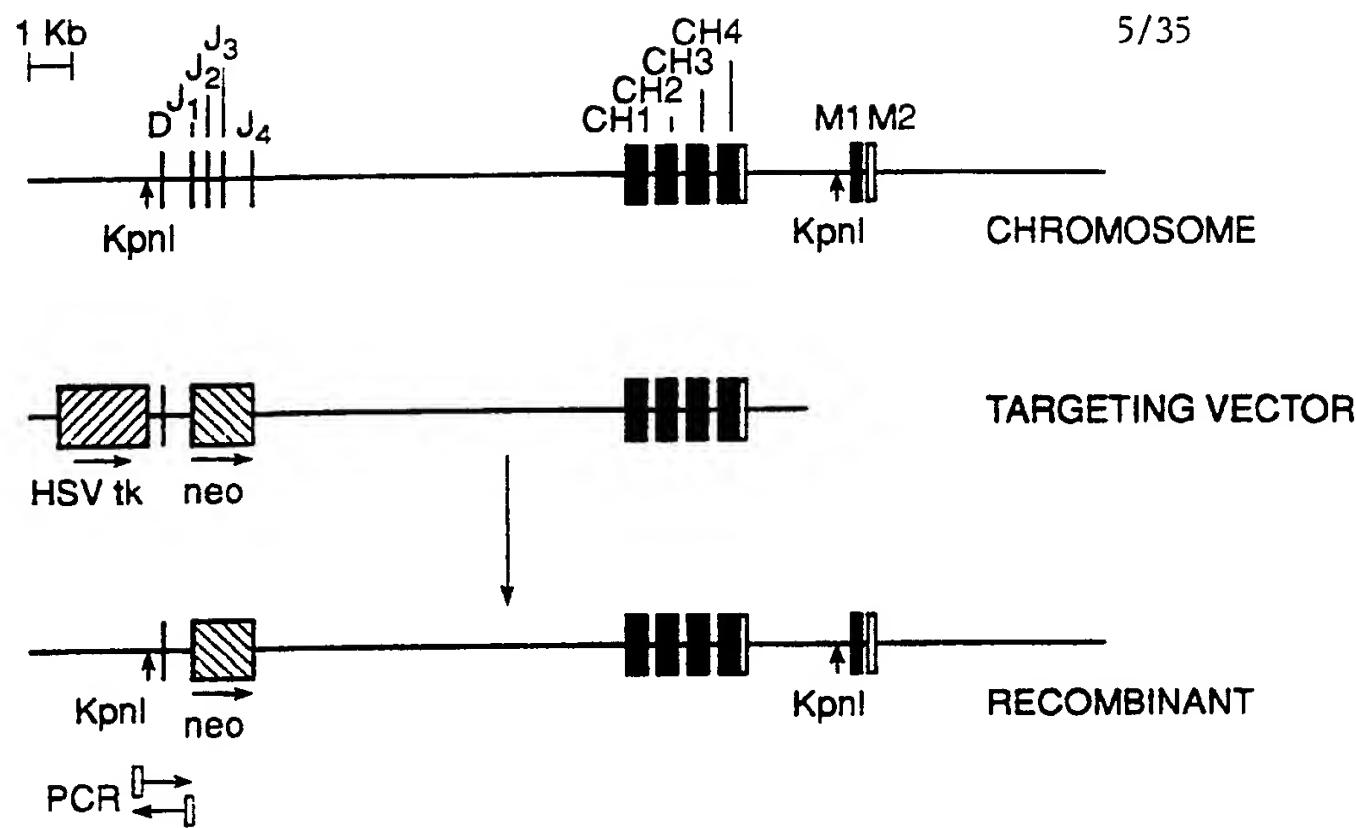
FIG. 1

FIG. 2

FIG. 3

FIG. 4

3/35

FIG. 5

FIG. 6

FIG. 7

FIG. 8

FIG. 9

FIG. 10

7/35

FIG. 11

FIG. 12

OLIGO-1	OLIGO-2	OLIGO-3
OLIGO-4	OLIGO-5	OLIGO-6

```
                                82 Xba I
                                78 EcoR V              115 Kpn I
                       60 Sty I                111 Sma I
                       60 Nco I         94 Sph I           127 Mlu I
                       56 BamH I      89 Xho I        121 HinD III
         42 Sfi I           75 Cla I        106 EcoR I    139 Not I
         40 Not I      65 BssH II       100 Bgl II    133 Spe I
           I             I  I    II    I      I I  II   I   I  II
```

147 BASE PAIRS
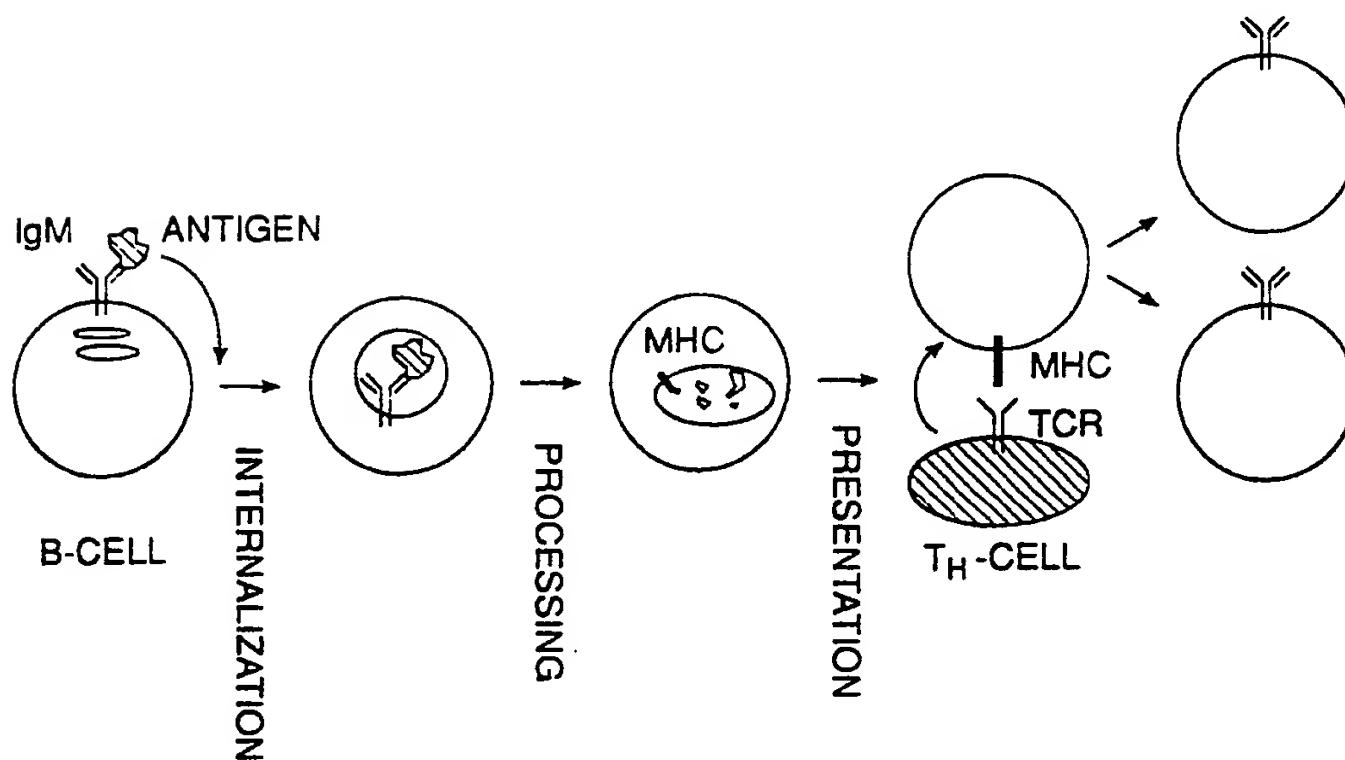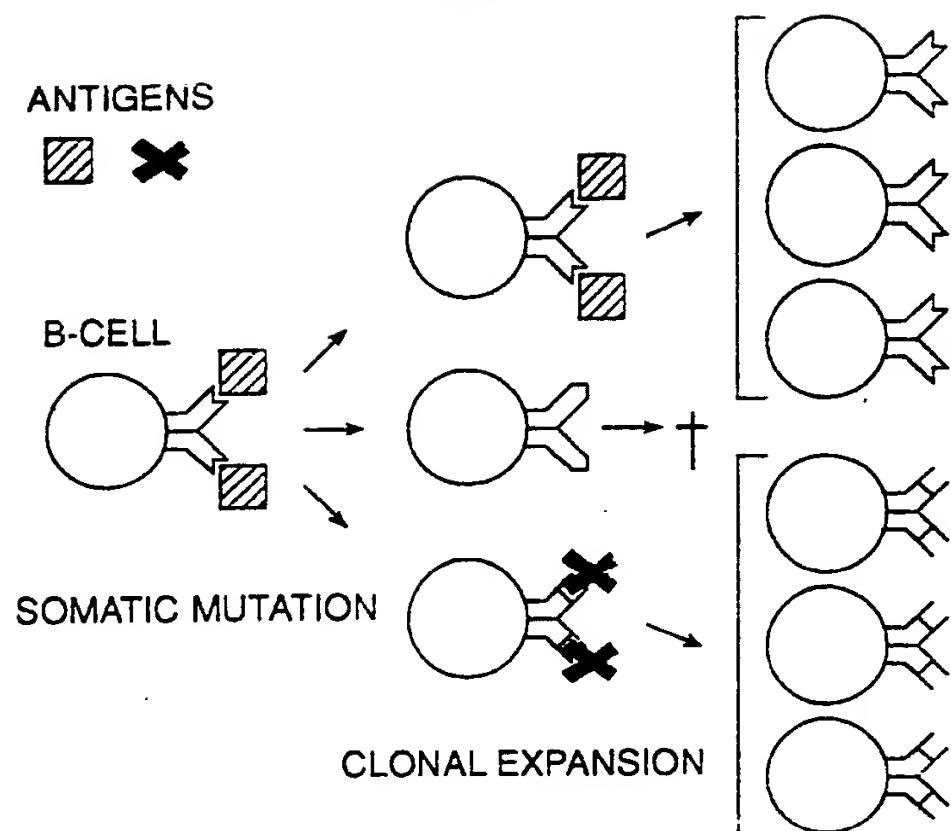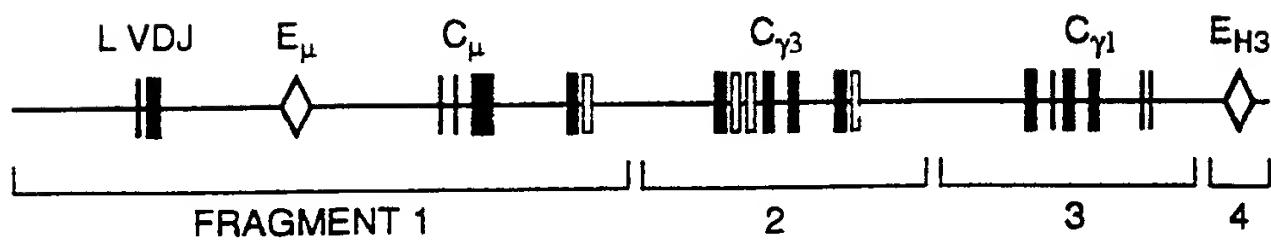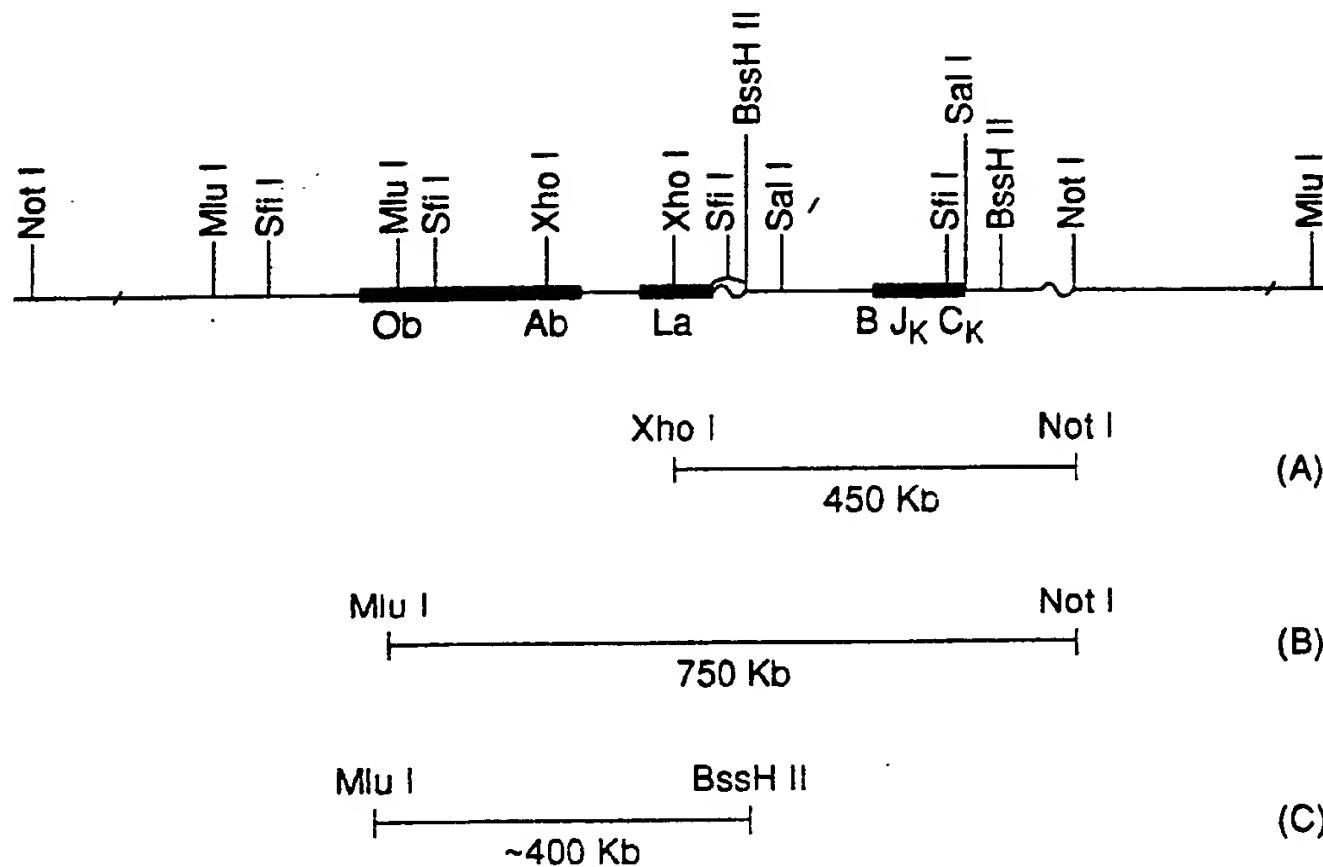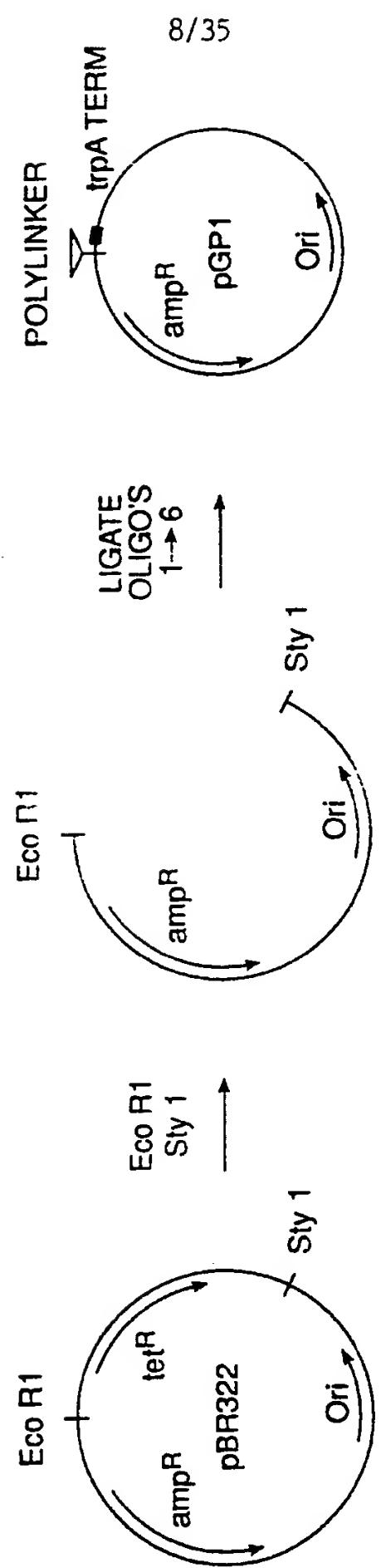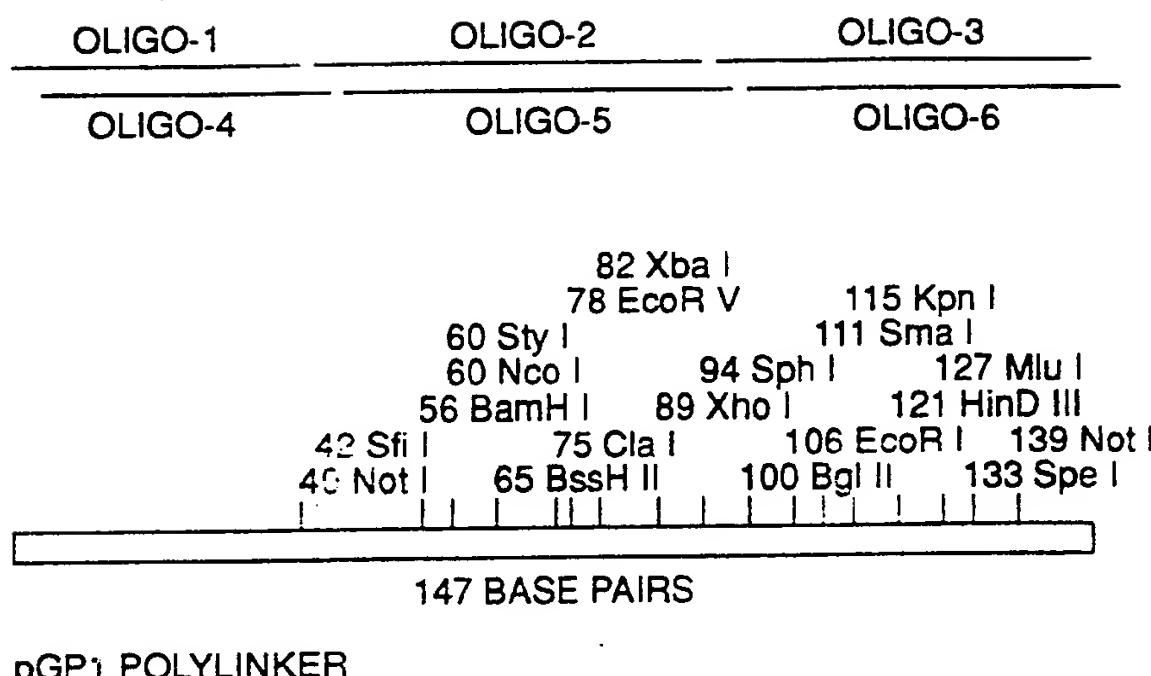
pGP1 POLYLINKER

FIG. 13

10/35

HUMAN μ LOCUS

FIG. 14

FIG. 15

HUMAN C$_{\gamma 1}$ GENE

FIG. 16

pHIG1/Sfi I
+
pREG2 Sfi I
INSERT

C_μ

pHI G3'

J_H

Sfi I

$C_{\gamma 1}$

3' ENH
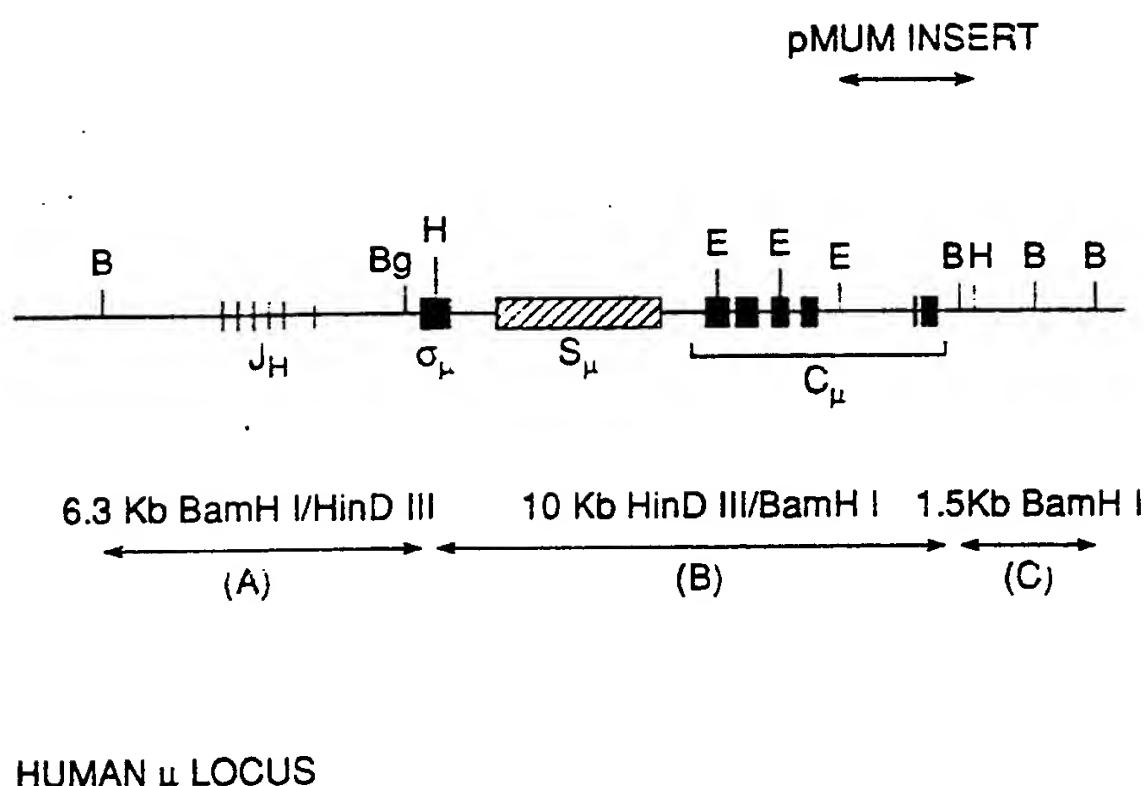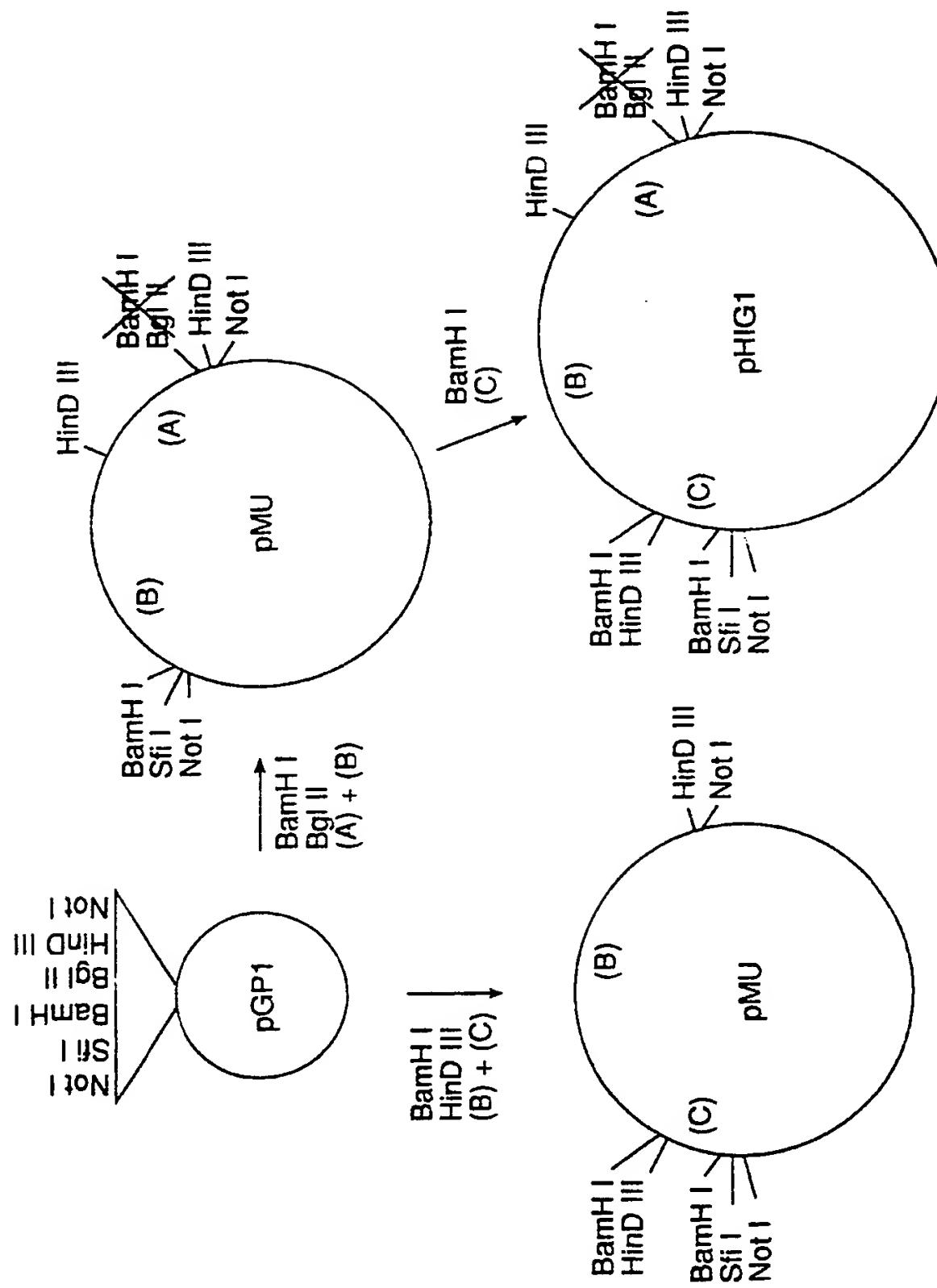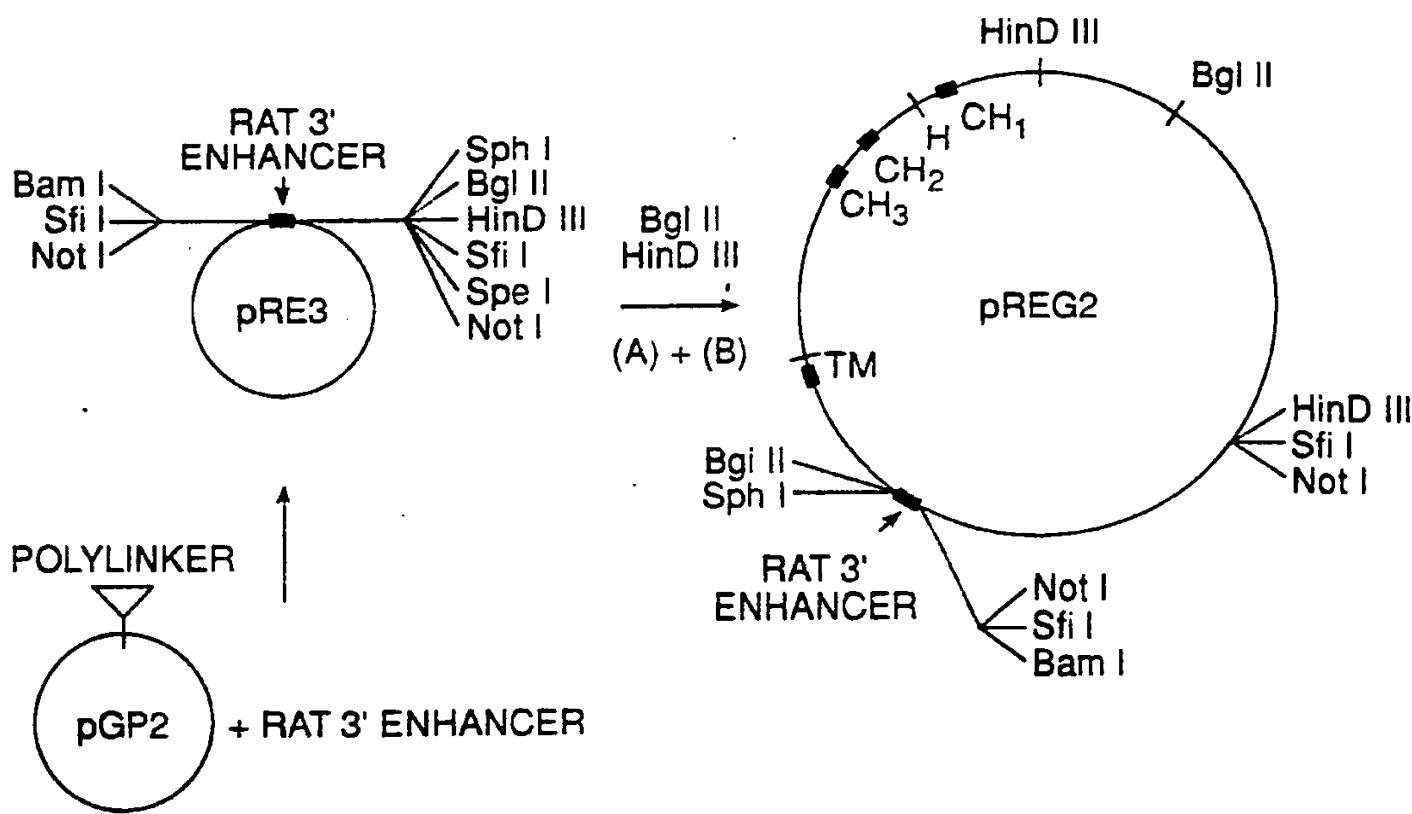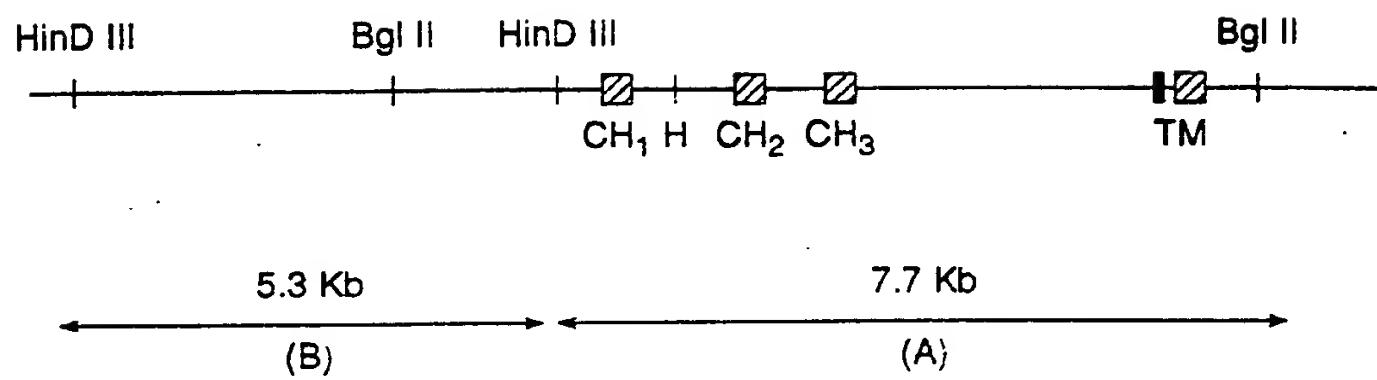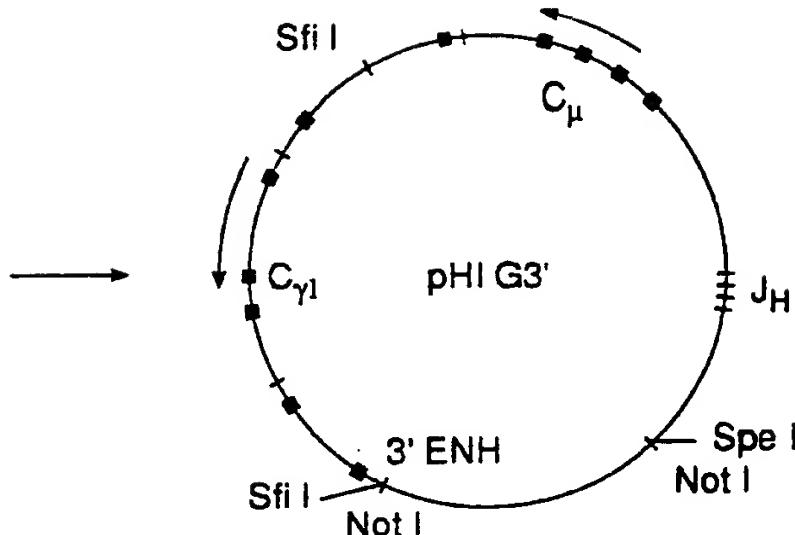
Sfi I

Not I

Spe I
Not I

31.5 Kb Not I/Spe I INSERT

pCON1/Sfi I
+
pREG2 Sfi I
INSERT

C_μ

pCON

Sfi I

$C_{\gamma 1}$

3' ENH

Sfi I
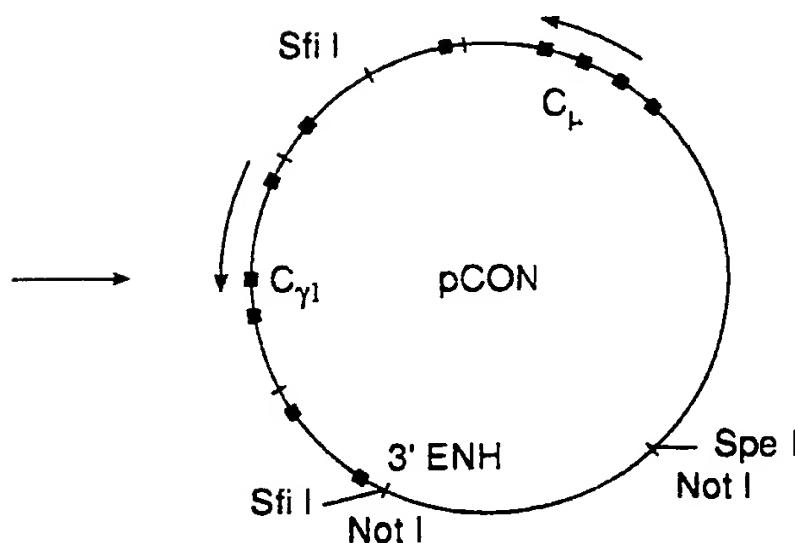
Not I

Spe I
Not I

25 Kb Not I/Spe I INSERT

FIG. 17
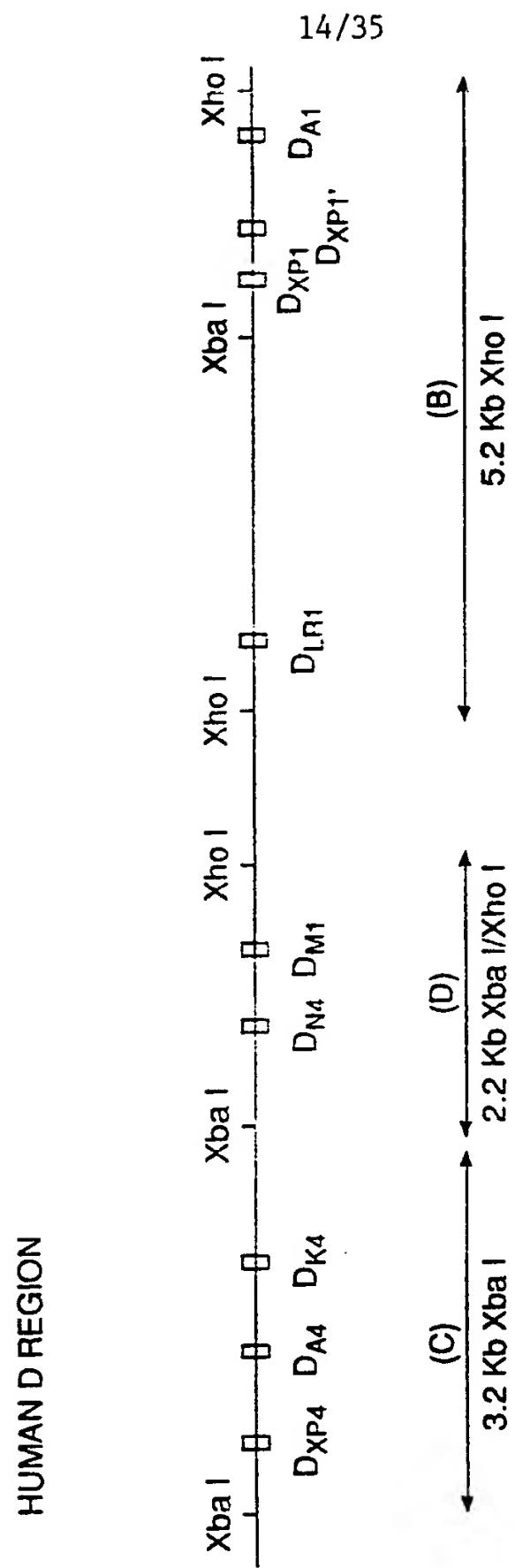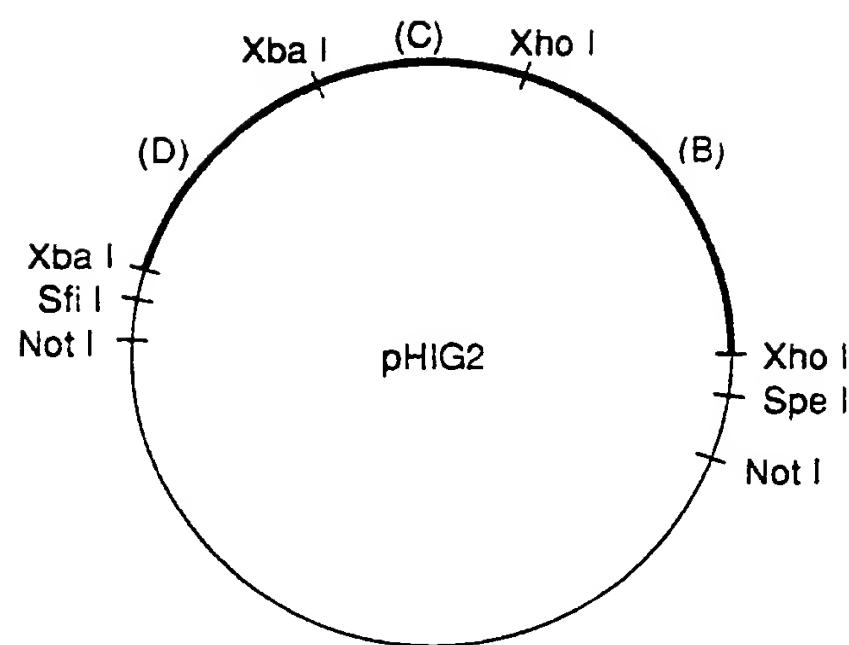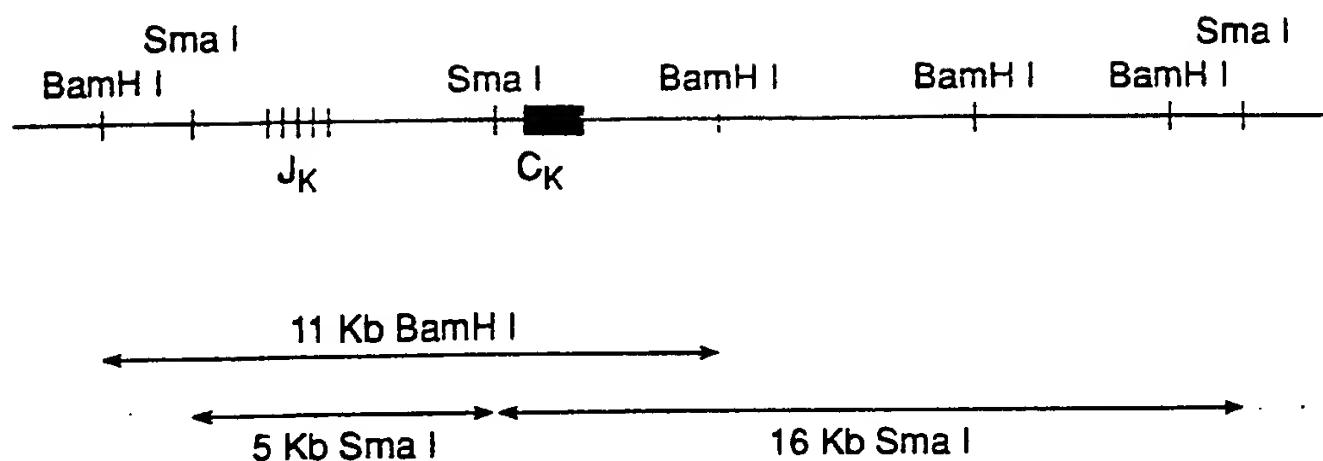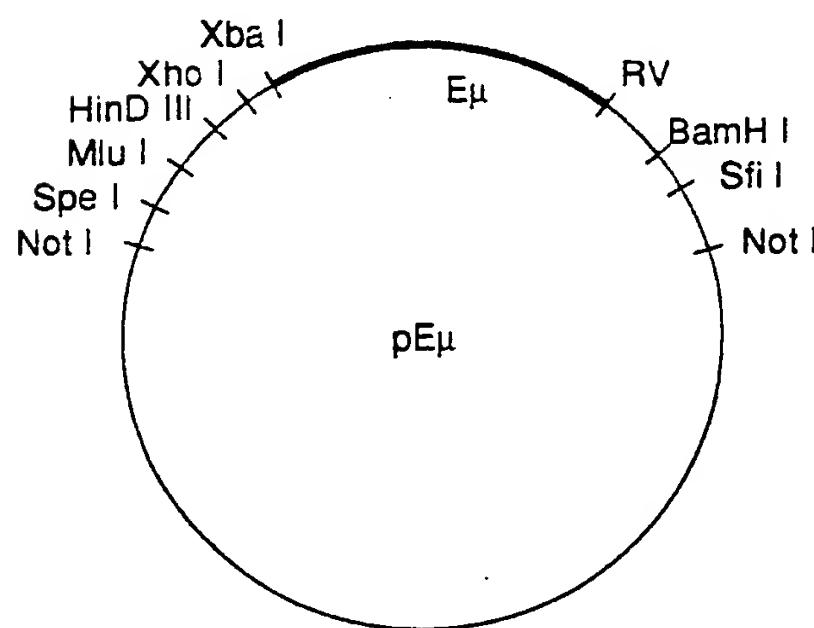
14/35

HUMAN D REGION

FIG. 18

FIG. 19

FIG. 20

FIG. 21

FIG. 22

MOUSE HEAVY CHAIN LOCUS

FIG. 23a

FIG. 23b

20/35

FIG. 23c

21/35
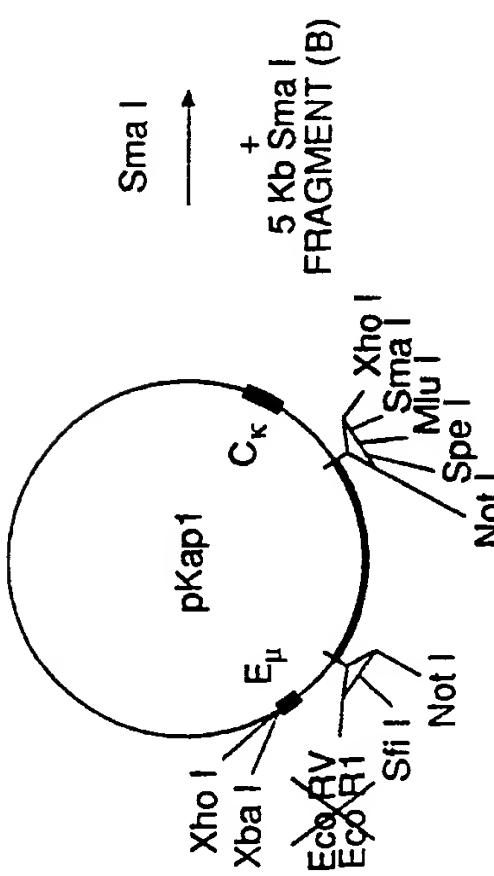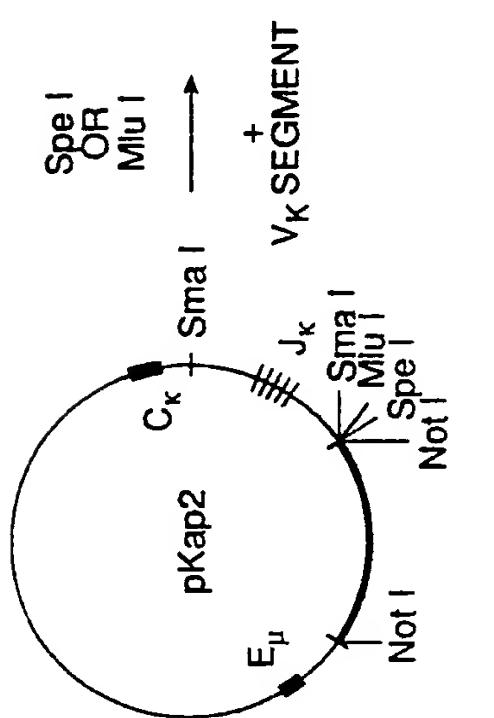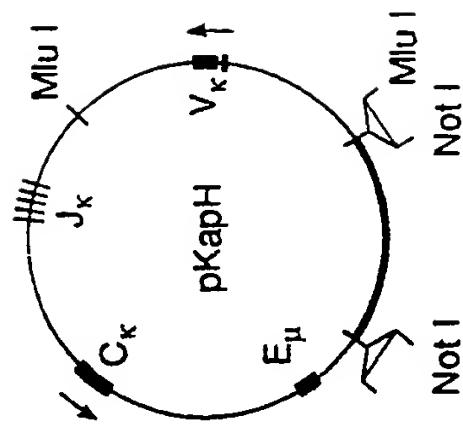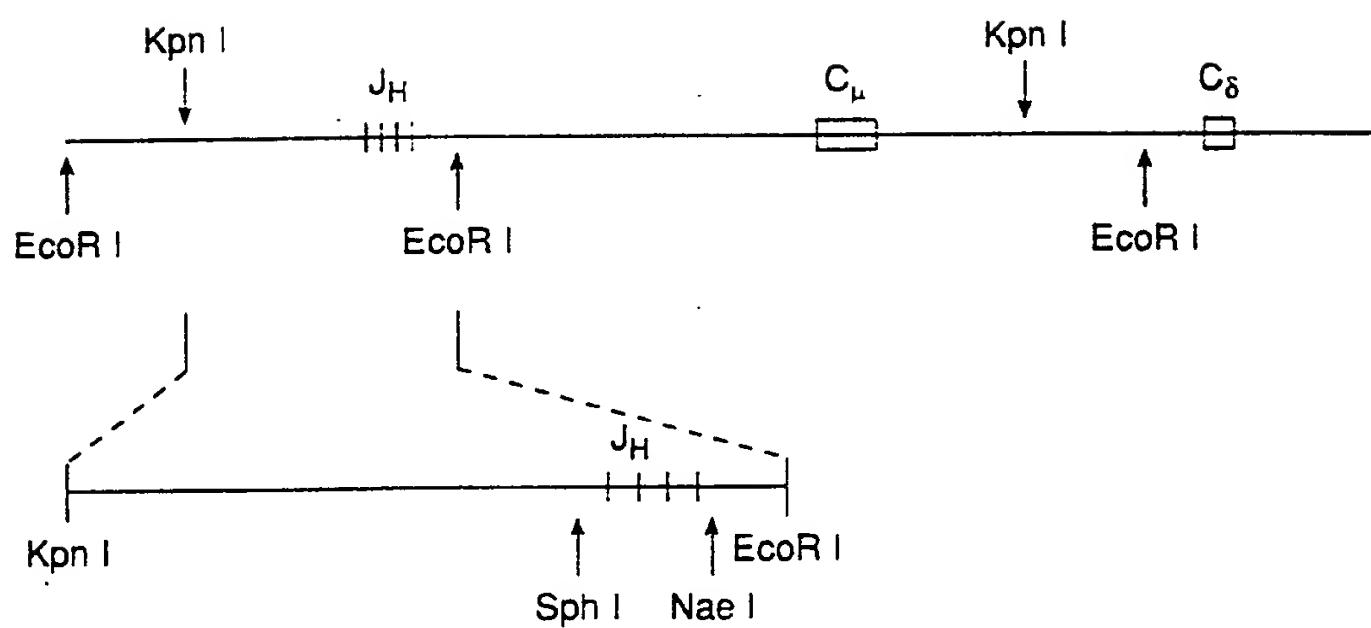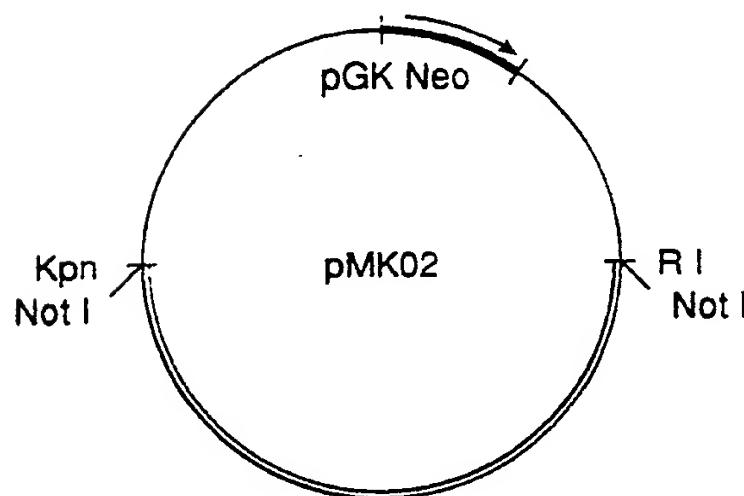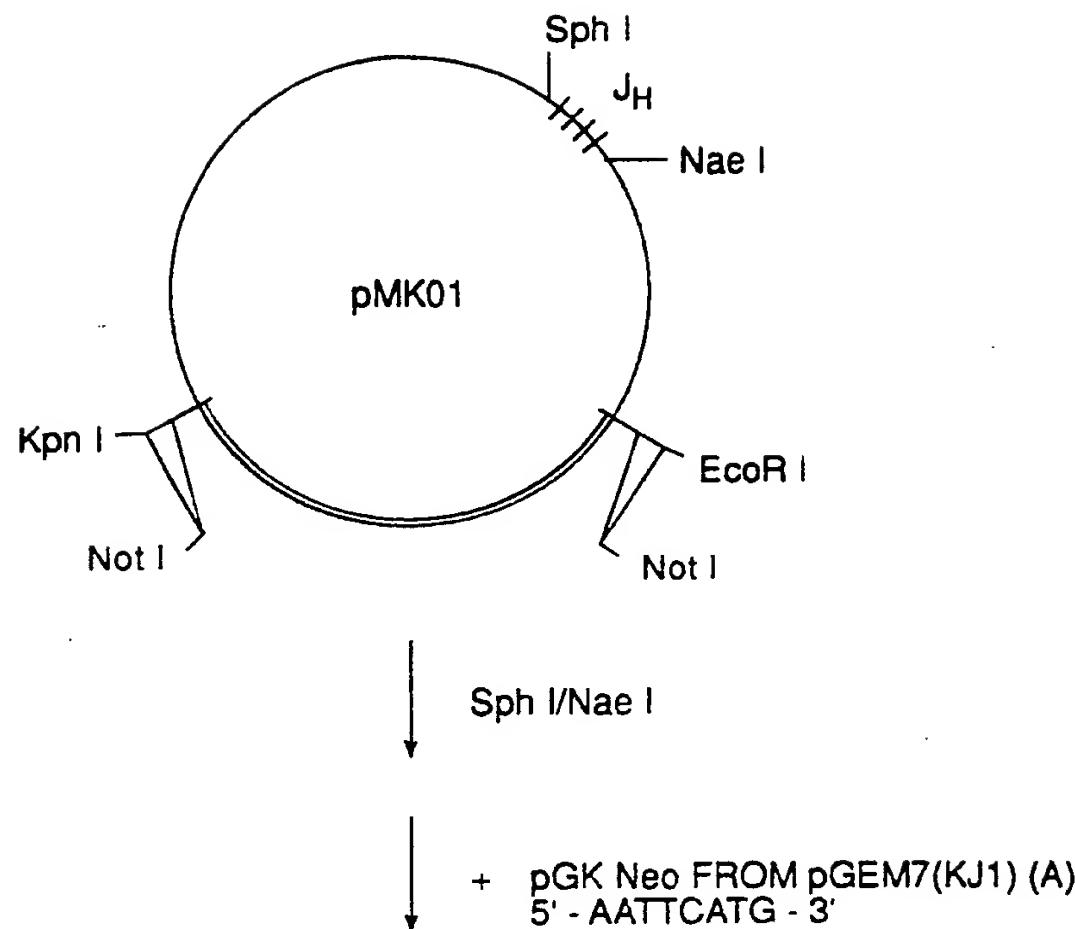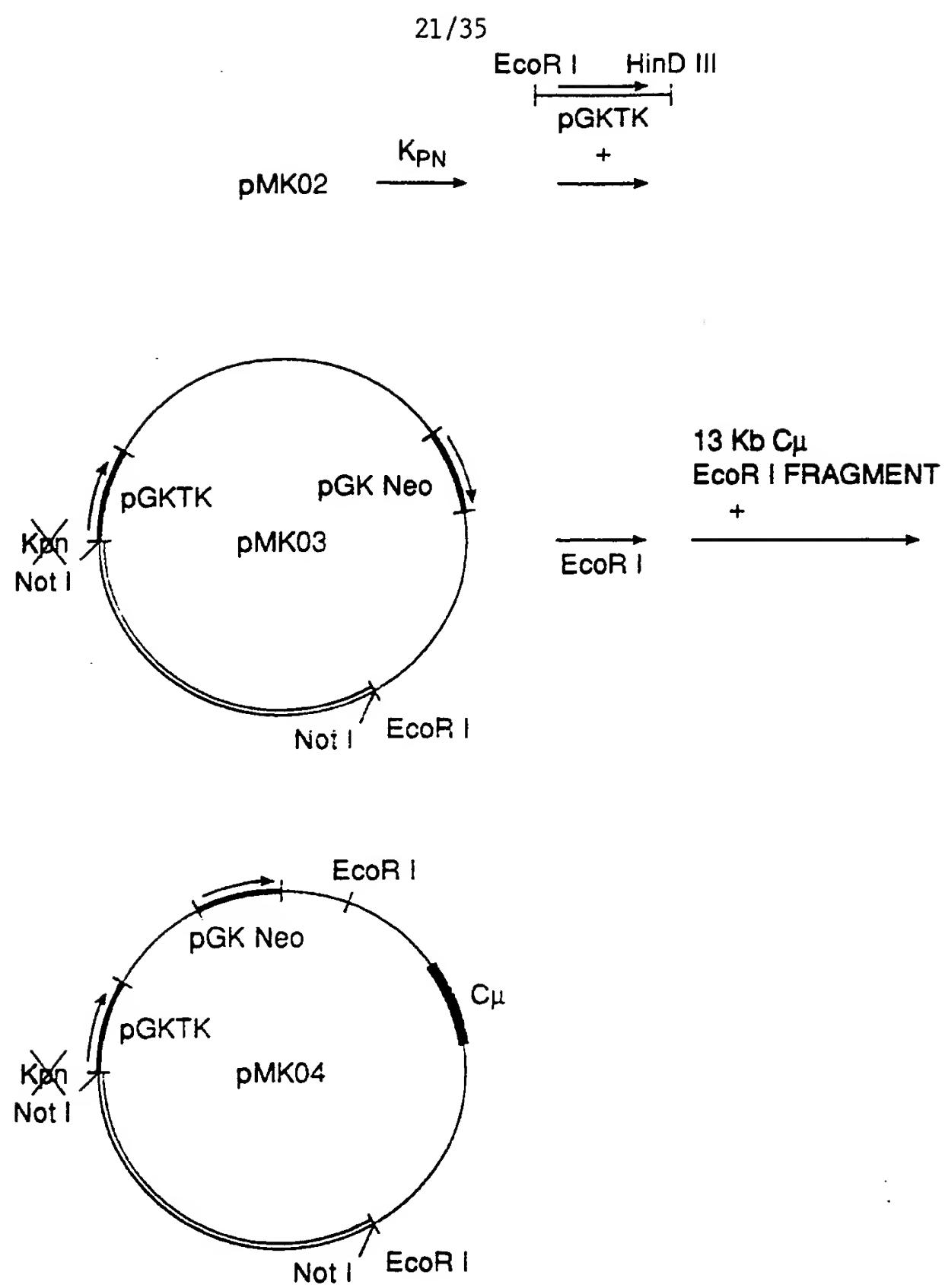
FIG. 23d

MOUSE KAPPA GENE

FIG. 24a

FIG. 24b

24/35

FIG. 24c

FIG. 25

KAPPA LIGHT CHAIN TARGETING VECTOR

A) GENOMIC KAPPA LOCUS

B) pNEO-K3'

C) pNEO-K3'5'

D) J/C K1

E) TARGETED KAPPA LOCUS

FIG. 26a

MOUSE HEAVY CHAIN TARGETING VECTOR

A) GENOMIC J_H LOCUS

B) puc18 J_H - NEO

C) pGMT-TK

FIG. 26b

D) pGMT-TK-J$_H$5'

E) J$_H$K01

F) TARGETED J$_H$ LOCUS

MOUSE HEAVY CHAIN TARGETING VECTOR

FIG. 27

29/35

FIG. 28

FIG. 29

FIG. 31

FIG. 32

33/35

FIG. 33

Not I   Xho I   J_K   C_K   Sal I   Not I

pJCK1

FIG. 34

35/35

SYNTHETIC HEAVY CHAIN VARIABLE REGION

FIG. 35

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

US: 800/2; 536/27

IPC(S): A01H 1/00; C07H 21/00

II FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
US:	800/2; 536/27

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

APS, CAS, BIO80

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[*]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	European Journal of Immunology, volume 19, issued 1989, Yasui et al., "Class Switch from μ to d is mediated by homologous recombination between γμ and εμ sequences in human immunoglobulin gene loci," pages 1399-14-3, see entire article.	75,77,78 83,84 107,115,116 119
Y	The EMBO Journal, volume 7, number 13, issued 1988, Ichihard et al., "Organization of human immunoglobulin heavy chain diversity gene loci", pages 4141-4150, see entire document.	1,6,25,27 75,115,116 117, 119 127,137,146

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 December 1991	08 JAN 1992

International Searching Authority	Signature of Authorized Officer
ISA/US	Suzanne Ziska

International Application No.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document. with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Nature, volume 342, issued 23 November 1989, "Germ line transmission of a disrupted B$_2$ macroglobulin gene produced by homologous recombination in embryonic stem cells", pages 435-438, see entire document, Zijlstra et al.	28,29,30,31
Y	Nucleic Acid Research, volume 15, number 23, issue 1987, Lorenz et al., "Physical map of the human immunoglobulin K locus and it implication for mechanisms of V$_K$-J$_K$ rearrangement", pages 9667-9676, see entire document.	10-15,25 27,29,30,32 34,35,108 111-115
Y	Proceedings National Academy Sciences, volume 86, issued September 1989, Bruggemann et al. " repertoire of monoclonal antibodies with human heavy chains from transgenic mice", page 6709-6713, see entire document.	A 1,2,4,6,7,8 9,25-27,32 34,35,75,76 78,79,81-84 86,107,109 110,114,116 117,119,127 128,129,130 131-138,146
Y	Nature, volume 314 issued 28 March 1985, Rusconi et al., "Transmission and Expression of a specific pair of rearranged immunoglobulin µ and K genes in a transgenic mouse line", pages 330-334, see entire document.	1-4,6-8, 10-14,25-27 33,35,75,76 79,107-115 116-119,130 137,138

International Application No.

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET	
Y	Proceedings National Academy Science, volume 83, issued April 1986, Yamamurd et al., "Cell type specific and regulated expression of human γ1 heavy chain immunoglobulin gene in transgenic mice", pages 2152-2156, see entire document	1,3-5,6-8, 25-27 33,75,76,80 81,85,107-110 114-117,119 127-129,137 138,146

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers _____ , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers _____ , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3.☐ Claim numbers _____ , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See Attached

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
1-15,25-35,75-86,107-119,127-123,137,138,146

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 11-87)

Box 4.C. (Cont'd)

The present application is a Continuation-in-Part of USSN 575,962 filed 31 August 1990, which is a C-I-P of USSN 574,748 filed 29 August 1990.